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EFFECTS OF VISIBLE RADIATION ON SALMONOID EMBRYOS AND LARVAE[1]

RONALD EISLER[2]

University of Washington, College of Fisheries, Seattle 5, Washington

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INTRODUCTION

The biological impact of electromagnetic energy on living organisms has been the subject of many investigations. Fish, in particular, have frequently been utilized as experimental animals as they exhibit radiation effects that are similar or identical to effects seen in other vertebrate animals (Donaldson and Foster, 1957). A literature search indicates that energy effects of the visible portion of the spectrum on growth and development of aquatic animals has, to a large extent, been ignored. Accordingly, the present study seeks to answer the following questions: which developmental stages of chinook salmon *Oncorhynchus tshawytscha* are most sensitive to a single dose of high-intensity, visible radiation; and, what are the effects of graded intensities of visible rays on these light-sensitive stages. Criteria employed to evaluate effects of light on salmon include: mortality; growth in length and weight; number of dorsal fin rays, vertebrae, anal fin rays, and parr marks; ratio of head length in fork length; rate of development; number of monstrosities; and gross histology.

HISTORICAL REVIEW

Examination of available published material shows that both ionizing (*i.e.*, X-rays, ultra-violet) and non-ionizing (*i.e.*, visible light, infrared, radio waves) radiations can elicit similar symptoms in fish, possibly through entirely different causal mechanisms. However, if the two types of radiation are to be compared, the following variables must be considered: wave length and intensity of incident radiation,

[1] Contribution Number 138, College of Fisheries, University of Washington.
[2] Present address: U.S. Fish and Wildlife Service, Sandy Hook Marine Laboratory, Highlands, New Jersey.

duration of exposure, species irradiated, developmental stage exposed to radiation, and physio-chemical environmental conditions. The following review of selected papers illustrates the biological effects of these variables on fish, and permits a comparison of the visible portion with other components of the electromagnetic spectrum.

Roentgen Radiation

The effects of X-irradiation of early developmental stages of fish has been documented by several investigators. Welander *et al.* (1948) exposed "eyed" chinook salmon embryos to X-ray dosages ranging from 250 to 10,000r. High doses caused retardation of cutaneous pigment, destruction of hemopoietic tissue, reduction of fingerling growth, lesions, loss of vitality, paralyzing effects, edema, and death. Welander (1954) irradiated six different embryonic stages of rainbow trout and observed that earliest stages were most sensitive. Survivors of irradiated groups displayed impaired growth, a proportionately larger head, reduction in number of dorsal and anal fin rays, and fewer parr marks. Solberg (1938b) noted that *Fundulus heteroclitus* embryos X-rayed in early stages of development were more deformed after treatment than older embryos. Regions of highest sensitivity were tip of the head, tail, and heart; further, liver and pancreas failed to develop in embryos X-rayed before 40 hours. Solberg states that any degree of deformity can be produced by appropriate selection of the developmental stage and the dose. Eisler (1959) exposed three embryonic stages of the flatfish *Parophrys vetulus* to roentgen radiation. Death and spinal column deformities were higher in irradiated groups. A median lethal dose of 136r was computed at time of first feeding. Embryos of salmon, trout, perch, paradise fish, goldfish, and killifish are all sensitive to X-radiation (Ancel and Vintemberger, 1925; Corbella, 1930; Rushton, 1936; Tanaka, 1942; Allen, 1951; Rugh, 1954; Rugh and Clugston, 1955; Konno *et al.*, 1955; Allen and Mulkay, 1960).

Foster *et al.* (1949) irradiated adult rainbow trout three months prior to spawning. The spawning date of irradiated fish was not different from that of controls but these fish produced conspicuously abnormal embryos. Significantly increased rates of mortality existed for as long as six months after hatching in groups receiving 500 or more roentgens. The effect of X-rays on gonads of *F. heteroclitus* indicates that gametes are quite resistant to dosages up to 200,000r

(Rugh, 1954; Rugh and Clugston, 1955). Sex products of *Oryzias* and of *Lebistes*, however, are measurably affected by comparatively low doses (Samokhvalova, 1935; Solberg, 1938a).

Chinook salmon fingerlings reacted to X-radiation with a decrease in number of erythrocytes and hematopoietic cells in the kidney; increased dosages resulting in higher mortality and reduced growth (Bonham *et al.*, 1948). Rainbow trout fingerlings exposed to 500r exhibited a marked reduction in food intake and were relatively sluggish when compared to controls but feeding was normal by day 22. By the 315th day post-irradiation, controls had significantly higher ovary weights (Kobayashi *et al.*, 1957, 1958).

Eruption of melanophores has been produced in goldfish with X-rays (Smith, 1932a, 1932b). Melanophore production may cause general cutaneous melanosis which, when aggravated by secondary infections of the skin, may result in death.

Increased dosages of roentgen radiation on adult goldfish result in higher mortality, increased pigmentation, marked shrinkage and atrophy of all lymphoid tissue, pycnosis of surviving lymphocytes, increase in amount of fibrous tissues in the lymph-nodes, disappearance of lymphoid elements in the spleen, and marked proliferation of macrophages containing blood pigment (Ellinger, 1939, 1940a; Ellinger and Gross, 1941). Medulla oblongata destruction in goldfish by X-rays is demonstrated by Ellinger (1940b), Ellinger and Davison (1942), and Davison and Ellinger (1942). It is suggested that roentgen pigmentation may be associated with destruction of a nerve center located in the medulla oblongata.

Nearly mature adult rainbow trout displayed injuries proportional to X-ray exposure. Irradiated groups exhibited hemorrhage, necrosis, intestinal damage, and retarded growth in length and weight (Welander *et al.*, 1950).

Modification of the physical environment can alter the LD_{50}. Gros *et al.* (1959) note that an LD_{50} of 1800r is obtained when goldfish are irradiated at 18° C but a temperature of 3° C increases the number of fish surviving. Characid fish *Hyphessobrycon scholzei* irradiated in 25 percent heavy water with 2500 or 3000r and kept after irradiation in tap water are more damaged than *H. scholzei* exposed to irradiation in tap water (Haggqvist and Hevesy, 1958).

Ultra-violet Radiation

Hinrichs (1925) subjected early cleavage stages of *Fundulus* to ultra-violet radiation. Resultant larvae exhibited cyclopia, inadequate circulation, abnormal non-motile tails, and anterior twinning. Hinrichs maintains that regions of the egg which have highest metabolic activity are most easily affected; furthermore, early stages of development are more easily modified than later stages. Hinrichs and Genther (1931) demonstrate abnormal circulatory and nervous systems in *Fundulus* as a result of exposing eggs to ultra-violet radiation during early periods of development. Twins and double monsters were produced in quantity when eggs were irradiated during the thirty minute period immediately prior to first cleavage.

Haempel and Lechler (1931) noted that eggs and sac-fry of three species of trout, as well as those of the pike (*Esox lucius*), were easily destroyed when exposed to ultra-violet light. Recently-fertilized eggs were more resistant to light and showed fewer unfavorable effects than eyed eggs. The three species of trout eggs seemed to differ in light sensitivity, and brook-trout eggs were especially susceptible.

Bell and Hoar (1950) used ultra-violet radiation of 2800-3100 Angstron units to irradiate eggs and alevins of sockeye salmon (*O. nerka*). Irradiation of eggs in the later stages of development stimulated hatching. Premature alevins, which hatched a month before controls, were abnormal in several respects. It was observed that their vertebral columns curved downward in the heart region; growth was retarded; yolk was not utilized as rapidly as in controls; and pigmentation was delayed. Mortality was particularly high at hatching time and the hatching process was abnormal. Histological examination of irradiated alevins revealed changes in epidermal and fibroblastic layers of the skin. Heavy doses of radiation produced severe degeneration of the epidermis. Lower dosages inflicted less damage and recovery began in thirteen days. Internal organs were not visibly affected.

Dunbar (1959) observed that fingerling rainbow trout exposed to bright sunlight developed necrotic areas around the dorsal fin and behind the head, in addition to an increased mortality rate. Fingerlings exposed to artificial ultra-violet light developed lesions histologically identical to those of "sunburned" groups. Similar results were obtained with lake trout fingerlings (Allison, 1960).

Crowell and McCay (1930) noted that trout, in water 2 cm in depth, were killed by five daily exposures of three minutes each of ultra-violet rays.

Kostomarov (1933) found that carp growth was readily checked and that their bodies lost both organic matter and ash when subjected to ultra-violet light.

Hybrid goldfish exposed to mercury vapor lamp radiation respond by acceleration in melanophore production. Parental forms, however, do not develop melanophores as a result of the treatment (Goodrich and Trinkaus, 1939).

Visible Radiation

Effects of visible radiation on incubating fish eggs and larvae have been the subject of several investigations. Smith (1916) noted that eggs of both chinook and pink (*O. gorbuscha*) salmon incubated in light hatched more quickly than those incubated in darkness. Newly-hatched fry from eggs incubated in light absorbed their yolk-sacs more rapidly than fry from dark-incubated eggs. Fish reared in darkness weighed 11 percent, and 41 percent, more than the light-reared group by the time of first feeding, and after 8 months of feeding, respectively. Eisler (1958) exposed eggs and resultant larvae of chinook and of silver (*O. kisutch*) salmon to varying intensities of fluorescent light from 24 hours after fertilization until absorption of the yolk-sac. In groups exposed to high intensities (157 foot-candles) all died before hatching; groups exposed to lower intensities exhibited lower mortality rates. Larvae of light-reared groups hatched earlier and were smaller, more darkly pigmented, and slower to absorb their yolk-sacs than larvae incubated in near-darkness. Eisler suggests that both radiant and thermal energy have similar but independent effects on salmon embryos. Gibor (1958) reports that eyed sockeye salmon eggs exposed to high intensities of fluorescent light while in glass dishes with a *Chlorella* suspension exhibited no ill effects (7 percent mortality) but freshly-fertilized silver salmon eggs under similar conditions exhibited a mortality of 17 percent. Hamdorf (1960) exposed embryos of *Salmo irideus* at different embryonic stages to visible radiation. The trout embryo increased in sensitivity until the heart started action, after which time sensitivity rapidly decreased. Exposed embryos exhibited high mortality, premature hatching, and decreased fingerling growth in length

and weight. Hamdorf infers that the substance in trout embryos destroyed by light is probably a pigment or a lactoflavene. Lyubitskaya (1956) notes that embryos of trout and whitefish display reduced growth when exposed to light at gastrulation. During the tail-bud developmental stage, perch and smelt embryos are adversely affected by darkness, red light, and infra-red radiation. Kurz (1920) exposed eggs and larvae of the flatfish *Pleuronectes* and the pike *Esox* to white light, total darkness, and to light of 5 different colors. White and short-waved light enhance development of black and colored pigments while red, yellow, and green retard development of both. Complete darkness retards development of black pigment and completely suppresses development of colored pigments. Qasim (1955, 1959) found that larvae of *Blennius pholis* and *Centronotus gunnellus* under alternate periods of light and darkness became more active, grew more quickly, and survived longer than those kept under continuous illumination. Embryos of shad, chinook salmon, silver salmon, rainbow trout, and cod that were exposed to bright light hatch earlier than embryos incubated under normal conditions of subdued light (Page, 1885; Murisier, 1920; Dannevig, 1947; Anon, 1957; Eisler, 1958; Hamdorf, 1960).

Brown (1946) notes that brown trout exposed to 6 hours of illumination daily grew significantly better than fish of the same age which received 12 hours of daily light. Seguin (1957) observed that lake trout raised in the darkness of covered raceways have a much better rate of growth than fish raised without cover. Chinook salmon fingerlings exposed to light intensities of 88, 116 or 157 ft.-c. for a period of 12 weeks were 56-59 percent heavier and 41-42 percent longer than the dark-reared (0.02 ft.-c.) group. Weight-length relationship was found to be the same for all groups (Eisler, 1957). Characins *Astyanax mexicanus* can live for apparently indefinite periods in total darkness but under such conditions develop thyroid hyperplasia, alteration of body form (caused by the accumulation of subdermal fat), kidney degeneration, vertebral column distortions, reduction in gonad size, and pituitary alteration (Rasquin, 1949; Rasquin and Rosenbloom, 1954). However, blind characins from caves are still capable of responding to light (Breder and Rasquin, 1947). Young salmon and trout feed readily in absence of light and may locate food by means other than a light receptor (Hoar, 1942), but herring do not feed in darkness (Battle *et al.*, 1936).

Meristic characters of fishes are significantly influenced by light. McHugh (1954) observed that vertebral counts of the grunion *Leuresthes tenuis* are lower if more light was present during the first month of life than if light was subdued or absent. Sockeye salmon embryos exposed to approximately 6 ft.-c. of artificial light for 16 hours daily displayed fewer anal rays and vertebrae, higher mortality, and greater proportion of abnormalities than groups exposed to light for only 8 hours daily (Lindsey, 1958). Canagaratnam (1959) subjected sockeye salmon embryos to varying intensities and durations of incandescent light. Higher intensities (5.9 ft.-c.) and longer light duration (16 hours daily) cause accelerated hatch, faster yolk-sac absorption, higher frequency of vertebral abnormalities, fewer scales, and fewer fin rays. Dannevig (1932b) noted that cod raised in a salt-water pond had lower average vertebral count than cod from the nearby sea. Conditions were similar except for more light (shallower water), and higher temperatures in the pond. *Salmo salar* eggs raised under conditions of partial darkness had a greater average number of vertebrae than eggs incubated under conditions of total darkness or of full daylight (Vibert, 1954).

Bright light can affect metabolism by increasing rate of oxygen consumption in whitefish and blind cave fish (Merker, 1926; Schlagel and Breder, 1947), increasing swimming time of goldfish and salmon (Shaw *et al.*, 1938; Fields, 1957), and by inducing locomotor disorganization (Breder, 1942).

Relationship between visible radiation and pigmentation in fishes is well documented. Cunningham (1893, 1895) noted considerable pigmentation on the unpigmented side of flatfish when the ventral surface was exposed to reflected daylight. These results were corroborated by Osborn (1940a, 1940b, 1941a, 1941b, 1948) on flounders and on catfish. Blinded fishes when illuminated from below while in dark tanks or from above while in white tanks also develop pigment. Extirpation of the pituitary gland of catfish results in melanophore degeneration which suggests that a pituitary hormone is indispensable to development and maintenance of melanin in melanophores. Sumner (1939, 1940a, 1940b) and Sumner *et al.* (1933, 1937, 1938) demonstrate that number of melanophores is directly proportional to darkness of the background; the darker the background, the greater number of melanophores. Intensity of incident light, above a rather low

level, had little if any effect upon number of melanophores or melanin content of *Lebistes* and *Gambusia*. However, marine gobies subjected to illumination of 65 ft.-c. for 87 days were chemically determined to have more melanin than fish kept at 1.5 ft.-c. intensity. Melanophore production in *Fundulus*, trout, minnows, and goldfish is demonstrated to be a function of light (Murisier, 1920; Connolly, 1925; Brown, 1936; Odiorne, 1937; Rasquin, 1946).

Some investigations have indicated that light plays a relatively unimportant role regarding the reproductive cycle of *Gambusia* (Medlen, 1951), fecundity of green throat darters (Hubbs and Strawn, 1957), number of broods per 100 days in guppies (Dildine, 1936), and activation of *Fundulus* testes (Burger, 1939; Mathews, 1939); however, most of the studies note that light definitely affects the reproductive cycle. Baggerman (1957) notes that rate of gonadal development of sticklebacks increases with day length, both at high and low temperatures. Light intensity of 300 ft.-c. does not accelerate gonadal development any more than 25 to 30 ft.-c., but 15 ft.-c. intensity may retard the maturation process. Baggerman states that sudden increase of light has a much stronger accelerating effect on maturation than gradual increase. Controlled light (gradually increasing light over normal daylight and then gradually decreasing light below normal daylight for a 2-3 month period) has been used successfully to modify the sexual cycle of brook trout *Salvelinus fontinalis*. Eggs, taken approximately 3 months earlier than usual spawning time for this species, were smaller than those of control fish but did not display differential mortality (Hoover and Hubbard, 1937; Hazard and Eddy, 1951; Corson, 1955). Spawning of adult sockeye salmon was delayed by lengthening daily light exposure; conversely, spawning was advanced by shortening daily light exposure (Combs *et al.*, 1959). Mortality of eggs taken from accelerated groups was significantly greater than that of controls. A more comprehensive treatment of effects of light on the reproductive cycle and endocrine organs of fishes is documented by Pickford and Atz (1957).

Attractant or repellent effects of light on fish have been studied extensively. Stuart (1953) states that emergence of brown trout alevins from gravel appears to be controlled by negative reaction of the alevin to light which is reversed suddenly just before the fish is ready to feed. Hoar *et al.* (1957) reports that Pacific salmon fry

are either positively phototactic (chum and pink salmon); negatively phototactic (sockeyes); or indifferent to light of moderately high intensities (silver salmon). Reaction to light has been observed in larvae of salmon, trout, cod, herring, eels, and bream (Dannevig, 1932a; Dannevig and Sivertsen, 1933; Privol'nev, 1956; Woodhead, 1957). Literature regarding light-guiding of salmonoids has been reviewed by Fields (1957). He states that downstream migrants of all five species of Pacific salmon as well as steelhead trout could be guided by use of light barriers, moreover, yearling salmonoids also avoided lighted areas. Fields indicates that the number of adult salmon ascending a fish ladder is significantly higher during night and greater during dark nights than light nights; further, dark-adapted salmon avoided light barriers up to 40 ft.-c. intensity but fish adapted to light at various environmental intensities did not exhibit marked negative phototaxis. Juvenile and adult herring, mackeral, whiting, suckers, mudminnows, lampreys, and cod also respond to light (White, 1919; Hineline, 1927; Gribble, 1934; McKenzie, 1935; Johnson, 1939; Andrews, 1947; Krefft and Schubert, 1950; Maeda, 1951; Sorensen, 1951; Schuler and Krefft, 1951; Lowe, 1952; Richardson, 1952; Kawamoto and Niki, 1952; Dragesund, 1958).

Infra-red Radiation

Experiments by Duncan (1956) indicated that silver salmon fingerlings could not perceive, or be affected by, infra-red radiation. Fields *et al.* (1955) aver that salmonoids could not be guided with infra-red lamps.

Radio Waves

At present, little quantitative work exists on effects of radio waves on animals. However, Shinn (1958) states that radiation from a powerful transmitter might cause heating of body tissues, and this, if excessive, would be harmful.

Preliminary Experiment: Effects of High-Intensity (157 Foot-Candles), Visible Radiation on Different Developmental Stages of Rainbow Trout

Equipment

All studies were conducted in a windowless room located at the University of Washington College of Fisheries. Cool white fluores-

cent tubes (stock number F96T12), each 2.44 meters in length, were used as experimental radiation sources. Spectrographic analysis of light emitted from each tube after it had passed through a sheet of window glass 3 mm thick revealed wave length range of 3662 to about 7000A with mean energy output of approximately 5600A (green-yellow). Energy contributed by various components of experimental light to total transmission, as measured at the water surface, is presented in Table 1. Spectrographs taken during the course

TABLE 1
ENERGY EMISSION, IN ARBITRARY COLOR BANDS, OF THE EXPERIMENTAL LIGHT SOURCE

Color band	Angstroms	Percent of total transmission at surface
Ultra-violet	less than 3800	0.57
Violet	3800 to 4300	7.72
Blue	4300 to 4900	21.19
Green	4900 to 5600	25.16
Yellow	5600 to 5900	18.63
Orange	5900 to 6300	18.10
Red	6300 to 7000	8.63
		100.00

Total energy (watts per 157 foot-candles) = 2.134.

of experiments showed no change in either wave length range or mean. To provide different light intensities, a fluorescent fixture for one tube, two tubes, four tubes, or none was employed. Tubes were placed 100 mm above the water surface and 175 mm from the zygotes. A plate of window glass 3 mm thick placed between light source and fertilized eggs, served to eliminate ultra-violet rays. In addition to the glass, several thicknesses of ordinary metal window screen were employed to control light intensity and to eliminate radio waves. All glass plates were cut from the same strip and all screens cut from the same roll of screening. Light intensity that each group received was measured in foot-candles by a Photovolt meter, model 210A. Light intensity measurements were made 175 mm from the source and were uncorrected for turbidity and reflectivity of water. Although large quantities of sediment in the water may significantly shift energy transmission peaks, data were not gathered on this subject; however, it was observed that heavy sedimentation did not occur.

Cement hatchery troughs, 4.24 meters in length, served as incubating receptacles for zygotes, resultant larvae and fingerlings.

Troughs were painted aquamarine and coated with non-toxic clear paint (Tropalite). Stainless steel plates subdivided each trough into compartments. Plates were arranged in baffle pattern so that zygotes were assured of constant flow of water about them. In order to insure uniform exposure of fertilized eggs to light, glass rod trays were employed in preference to the usual metal incubating baskets.

Water flow of about 7 l./min. was maintained in each trough, water temperature varying with that of its source (Lake Washington). Water temperatures were recorded with a Bristol thermograph, and accuracy was verified daily by a thermometer calibrated to 0.05° C. Water temperatures at the inlet of each trough were identical at any one time but ranged from 8.6 to 14.0° C during the experiments. Temperatures at the outlet of each trough remained within 0.1° C of each other; however water temperatures at outlets were consistently about 0.4° C higher than at inlets. No attempt was made to maintain a constant temperature as salmon normally spawn when the water begins to cool and embryos develop normally during a period of falling temperature.

Methods

On March 5, 1958, eggs from 4 rainbow trout, *Salmo gairdneri*, of University of Washington brood stock were fertilized with milt from 2 different males. Physical characteristics of test animals are listed in Table 2. After 24 hours, opaque eggs were removed and the

TABLE 2
PHYSICAL CHARACTERISTICS OF RAINBOW TROUT USED IN PRELIMINARY EXPERIMENT

	Pair			
	A	B	C	D
Length of female (cm)	57	55	54	54
Length of males (cm)	58,57	60,56	54,55	61,53
Diameter of eggs (mm)	5.5	5.3	5.0	5.2

All fish were in the 2 + age group.

remainder distributed among cement troughs as indicated by Fig. 1. At various developmental stages, a different group was exposed to an intensity of 157 foot-candles of the experimental light source for a continuous 72 hours. During each irradiation period, nonexposed groups received less than 0.02 foot-candles of reflected light. Groups were examined at the beginning and end of each three day exposure

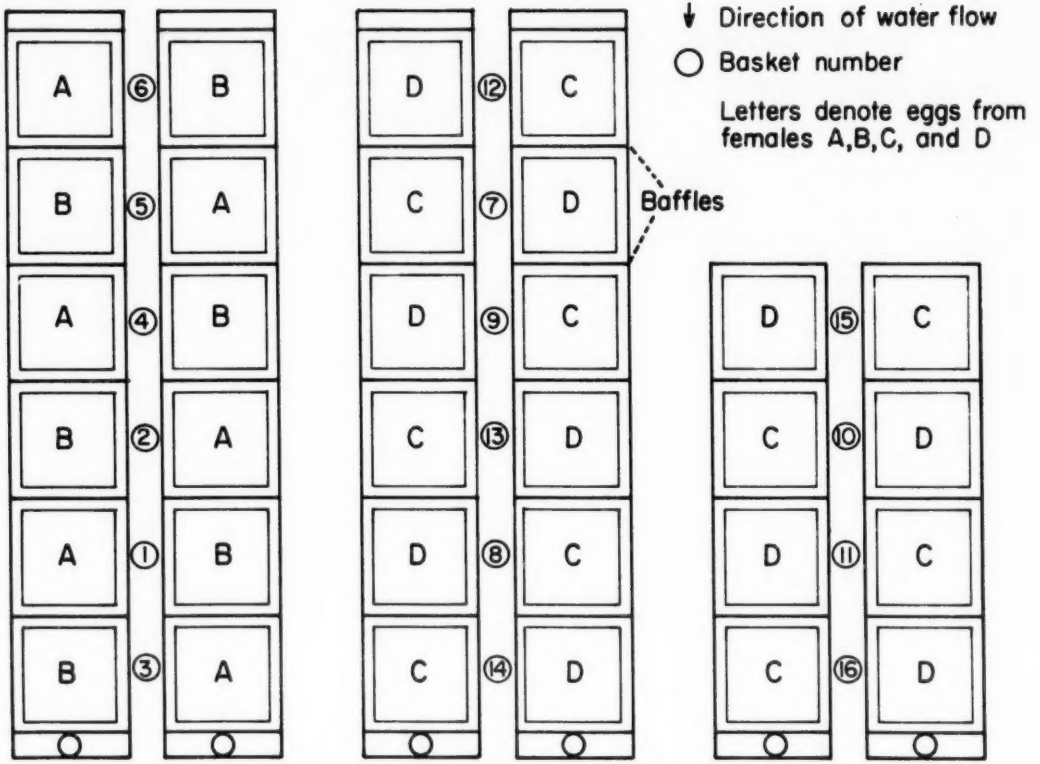

FIGURE 1
Diagram of arrangement of egg baskets in Williamson troughs in experiment on effects of light on rainbow trout eggs.

period, at which time zygotes received approximately 8 foot-candles of artificial light for 2 to 3 minutes. During inspection all opaque eggs were removed and preserved in Stockard's[3] solution so that the developmental stage at death could be ascertained.

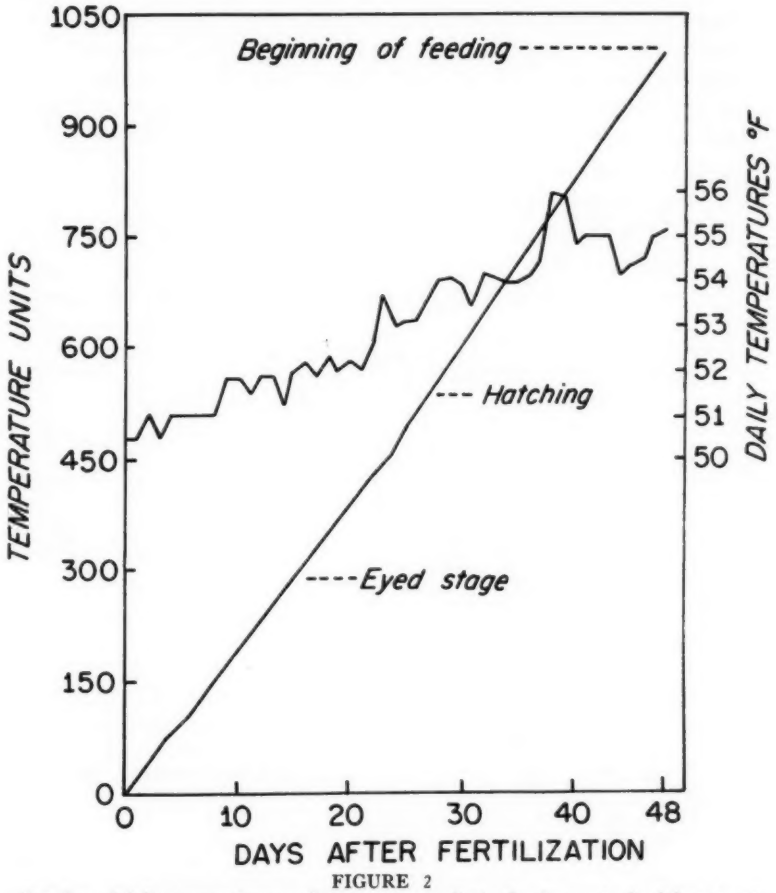

FIGURE 2
Relation of daily temperatures and temperature units to development of rainbow trout.

[3] Fifty parts of 40 percent formalin, 40 parts of glacial acetic acid, 60 parts of glycerine and 850 parts of water.

The following five developmental stages were arbitrarily adopted:

Stage I *Eggs in which eyes of embryos have not become pigmented nor have fin rays appeared.* This period extends from fertilization through closing of the blastopore.

Stage II *Eggs in which eyes of embryos are pigmented.* During this period, embryos continue to develop structurally until hatching.

Stage III *Embryos which are hatching.* This stage is usually characterized by eruption of yolk material through a single, small hole in the chorion.

Stage IV *Larvae, during absorption of the yolk-sac.* This covers the period between hatching and first feeding.

Stage V *Fry, after absorption of the yolk-sac.* Fish dying during this period are usually grossly abnormal or fail to feed.

The observed relationship between temperature and temperature

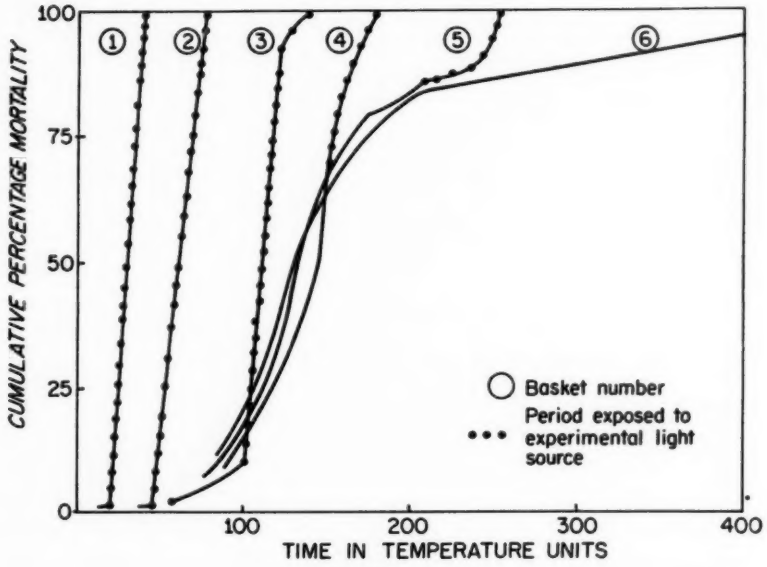

FIGURE 3

Cumulative percentage mortality of Pair A rainbow trout eggs after exposure to a light intensity of 157 foot-candles.

units[4] to development of rainbow trout under normal hatchery lighting conditions, is presented in Fig. 2.

Results

A summary of observations on effects of light on different developmental stages of rainbow trout is presented in Tables 3 to 6 and Figures 3 to 6. In general, groups exposed to 72 hours of continuous radiation during early embryonic stages (Tables 3 and 4, Figures 3 and 4) exhibit increased and earlier mortality when compared to controls. Embryos reaching stage II (eggs in which eyes of embryos are pigmented) in any light-exposed groups are significantly fewer than those of the control group (chi-square = 9.1**[5] for Pair A; 107.8** for Pair B).

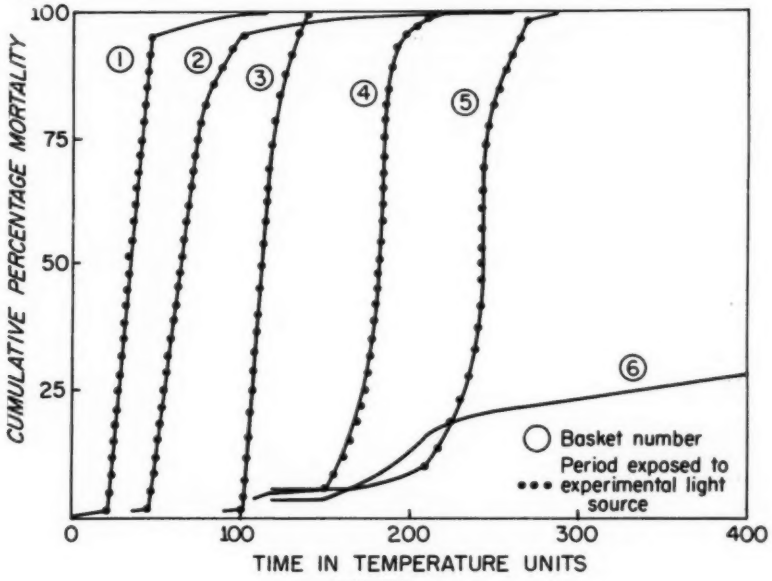

FIGURE 4

Cumulative percentage mortality of Pair B rainbow trout eggs after exposure to a light intensity of 157 foot-candles.

[4] The temperature unit theory described by Wallich (1901) states that one temperature unit is equal to one degree over 32° Fahrenheit for 24 hours.

[5] ** significant at the one percent level.
* significant at the five percent level.

Advanced embryonic and larval stages of trout irradiated after the 15th day are less sensitive than earlier stages (Tables 5 and 6, Figures 5 and 6). For example, Table 7 contains values from chi-square analyses of the hypothesis that there is no difference regarding the number of eggs that hatch among irradiated and non-irradiated groups. Results show that groups irradiated prior to formation of body pigments (7 and 8 of Pair C and 7 and 8 of Pair D) have significantly lower hatch than their respective controls. However, groups 9, 10, and 11 which were irradiated at later stages of development were, with one exception, apparently unaffected by light.

The high mortality of controls in these experiments precludes quantitative determinations of effects of light on developing embryos,

TABLE 3

SUMMARY OF OBSERVATIONS ON EXPERIMENT OF EFFECTS OF LIGHT ON DEVELOPMENTAL STAGES OF RAINBOW TROUT EGGS—PAIR A

(For description of developmental stages, see text.)

Basket number	1	2	3	4	5	6
Number of eggs in sample	400	400	400	400	400	400
Time of exposure						
in temperature units	19 to 47	47 to 101	101 to 139	151 to 209	209 to 268	control
in days	1–3	4–6	7–9	11–13	14–16	
Stage I						
Mortality	400	400	400	400	400	391
Percent	100	100	100	100	100	97.5
Stage II						
Mortality	—	—	—	—	—	6
Percent	—	—	—	—	—	1.5
Stage III						
Mortality	—	—	—	—	—	3
Percent	—	—	—	—	—	1
Total						
Hatch	0	0	0	0	0	0
Percent	0	0	0	0	0	0
Stage IV						
Mortality	—	—	—	—	—	—
Percent	—	—	—	—	—	—
Total						
Mortality	400	400	400	400	400	400
Percent	100	100	100	100	100	100

TABLE 4
SUMMARY OF OBSERVATIONS ON EXPERIMENT OF EFFECTS OF LIGHT ON DEVELOPMENTAL STAGES OF RAINBOW TROUT EGGS—PAIR B
(For description of developmental stages, see text.)

Basket number	1	2	3	4	5	6
Number of eggs in sample	400	400	400	400	400	400
Time of exposure						
in temperature units	19 to 47	47 to 101	101 to 139	151 to 209	209 to 268	control
in days	1–3	4–6	7–9	11–13	14–16	
Stage I						
Mortality	400	400	400	400	400	305
Percent	100	100	100	100	100	76
Stage II						
Mortality	—	—	—	—	—	23
Percent	—	—	—	—	—	6
Stage III						
Mortality	—	—	—	—	—	32
Percent	—	—	—	—	—	8
Total						
Hatch	0	0	0	0	0	40
Percent	0	0	0	0	0	10
Stage IV						
Mortality	—	—	—	—	—	23
Percent	—	—	—	—	—	6
Total						
Mortality	400	400	400	400	400	383
Percent	100	100	100	100	100	96

but uniform acceleration of death in all groups exposed to light leaves no doubt that an adverse effect is present. Despite the high control group mortality, this study demonstrates that exposure of trout embryos to 157 ft.-c. of the experimental light for a continuous period of 72 hours results in higher mortality than controls and an arrested state of development prior to death; further, later developmental stages (*i.e.* stages having more pigmentation) which were similarly exposed are not influenced.

TABLE 5

SUMMARY OF OBSERVATIONS ON EXPERIMENT OF EFFECTS OF LIGHT ON DEVELOPMENTAL STAGES OF RAINBOW TROUT EGGS—PAIR C

(For description of developmental stages, see text.)

Basket number	7	8	9	10	11	12	13	14	15	16
Number of eggs in sample	281	281	281	281	281	281	281	281	281	281
Time of exposure										
in temperature units	268	328	389	451	514					
	to	to	to	to	to		control			
	328	389	451	514	580					
in days	16–	19–	22–	25–	28–					
	18	21	24	27	30					
Stage I										
Mortality	217	199	204	216	192	201	203	206	209	208
Percent	77	71	73	77	68.5	71.5	72.5	73	74.5	74
Stage II										
Mortality	63	75	47	24	24	36	32	34	25	22
Percent	22.5	27	17	8.5	8.5	13	11.5	12	9	8
Stage III										
Mortality	0	3	8	13	30	16	17	19	12	18
Percent	—	1	2	4.5	11	5.5	6	7	4.5	6
Total										
Hatch	1	4	22	28	35	28	29	22	35	33
Percent	0.5	1	8	10	12	10	10	8	12	12
Stage IV										
Mortality	1	4	5	21	10	19	11	11	13	22
Percent	0.5	1	2	7.5	3.5	7	4	4	4	8
Total										
Mortality	281	281	264	274	256	272	263	270	259	270
Percent	100	100	94	97.5	91	97	94	96	92	96

TABLE 6

SUMMARY OF OBSERVATIONS ON EXPERIMENT OF EFFECTS OF LIGHT ON DEVELOPMENTAL STAGES OF RAINBOW TROUT EGGS—PAIR D

(For description of developmental stages, see text.)

Basket number	7	8	9	10	11	12	13	14	15	16
Number of eggs in sample	220	220	220	220	220	220	220	220	220	220
Time of exposure										
in temperature units	268	328	389	451	514					
	to	to	to	to	to		control			
	328	389	451	514	580					
in days	16–	19–	22–	25–	28–					
	18	21	24	27	30					

(*Continued on next page*)

TABLE 6 (*Continued*)

Stage I										
Mortality	180	182	180	194	195	180	182	186	183	181
Percent	82	83	82	88	89	82	83	85	83	82
Stage II										
Mortality	39	38	24	15	14	17	18	11	21	19
Percent	17.5	17	11	7	6	8	8	5	10	9
Stage III										
Mortality	0	0	7	6	5	12	10	9	5	6
Percent	0	0	3	3	2	5	5	4	2	3
Total										
Hatch	1	—	9	5	6	11	10	14	11	14
Percent	0.5	—	4	2	3	5	5	6	5	6
Stage IV										
Mortality	1	—	2	5	4	6	2	5	10	8
Percent	0.5	—	1	2	2	3	1	2	4.5	3
Total										
Mortality	220	220	213	220	218	215	212	211	219	214
Percent	100	100	97	100	99	98	96	96	99.5	97

TABLE 7

CHI-SQUARE ANALYSES OF THE NUMBER OF TROUT THAT HATCHED FROM IRRADIATED AND NON-IRRADIATED GROUPS

Group	Sample size	Number hatching	Chi-square value (group—vs. controls)
Pair C			
controls[6]	1405	147	——
7	281	1	29.88**[5]
8	281	4	23.47**
9	281	22	1.80
10	281	28	0.06
11	281	35	0.97
Pair D			
controls[6]	1100	60	——
7	220	1	10.40**
8	220	0	12.57**
9	220	9	0.69
10	220	5	3.96*[5]
11	220	6	2.83

[6] No difference was found among the five Pair C control groups ($\chi^2 = 0.10$) and the five Pair D control groups ($\chi^2 = 0.05$) regarding the number of eggs that hatch; accordingly, each pooled group of five was treated as a unit.

* Significant at the 0.05 level; ** significant at the 0.01 level.

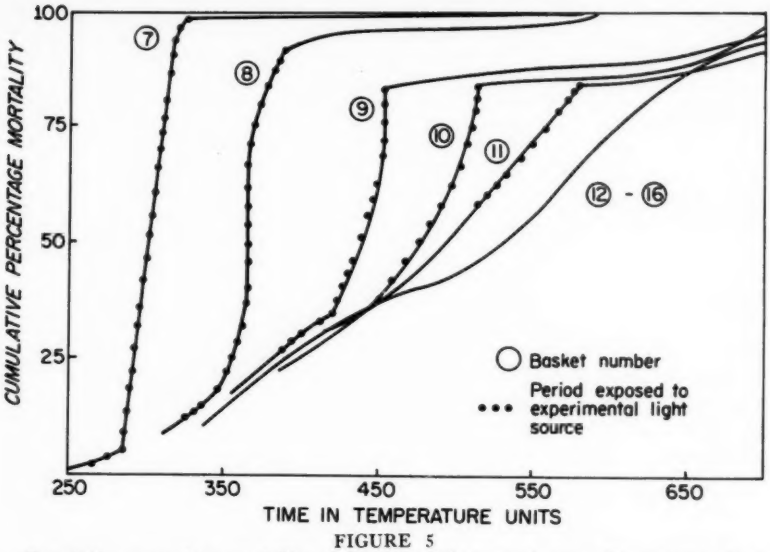

FIGURE 5

Cumulative percentage mortality of Pair C rainbow trout eggs after exposure to a light intensity of 157 foot-candles.

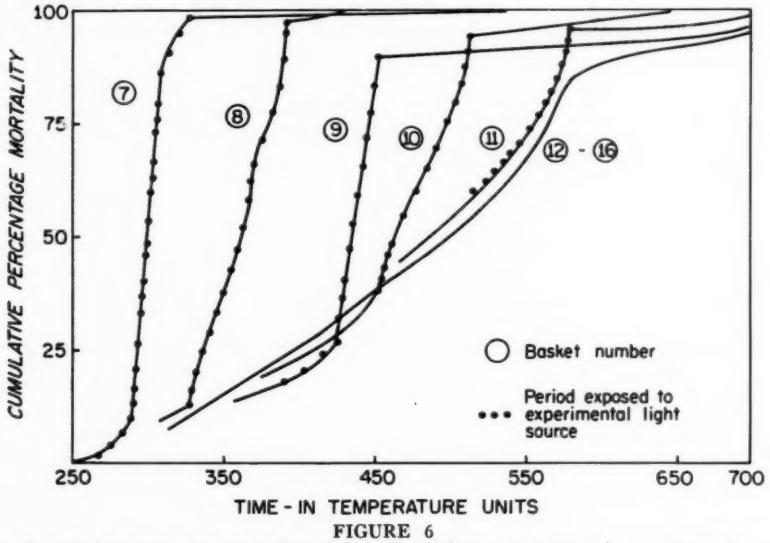

FIGURE 6

Cumulative percentage mortality of Pair D rainbow trout eggs after exposure to a light intensity of 157 foot-candles.

Experiment I: Effects of High-Intensity (157 ft.-c.), Visible Radiation on Different Developmental Stages of Chinook Salmon

Methods

On Oct. 10, 1958 eggs were taken from two chinook salmon at the Washington State Department of Fisheries' hatchery located in Issaquah. Eggs from each female (Pair A and Pair B) were fertilized with milt from a single male and incubated for 24 hours before distribution among cement troughs (Fig. 7). As with rainbow trout zygotes, each group was exposed to 157 ft.-c. of the experimental light source. However, salmon were exposed for a longer period (168 hours) of continuous radiation than trout (72 hours). During each exposure period, non-irradiated groups received less than 0.02 ft.-c. of light. Trays were inspected every 48 hours at which time opaque eggs were removed and preserved in Stockard's solution. During inspection time, which varied between 2 and 3 minutes, groups received about 6 ft.-c. of light.

Growth of chinook salmon over a period of time expressed as temperature units, as observed by the author, is presented in Table 8. These data are similar to other published information (Riddle, 1917; Wales, 1941; Battle, 1944) on salmonoid development. Attempts to classify dead embryos to exact stage of development at death were unsuccessful owing to embryonic decomposition between sampling periods; therefore, arbitrary stages described in the preliminary trout experiment were again utilized.

Time until hatching, in temperature units, was recorded for each group. At age 92 days, after larvae had absorbed their yolk-sacs and commenced feeding, lots were reduced to 123 fingerlings. At this time, 25 surplus fingerlings from each group were preserved for parr mark counts. Between age 92 and 197 days, salmon were fed a surfeit of raw, ground beef liver twice daily. During each feeding, fish were exposed to 8-10 ft.-c. of fluorescent light for 20 minutes. Uneaten liver and waste products were removed daily during the afternoon feeding period. Dead fish were removed daily and preserved in three percent formalin. Fingerlings were not exposed to any intensity greater than 0.02 ft.-c. except during feeding and measuring.

Weights and lengths of 50 fingerlings from each lot were taken

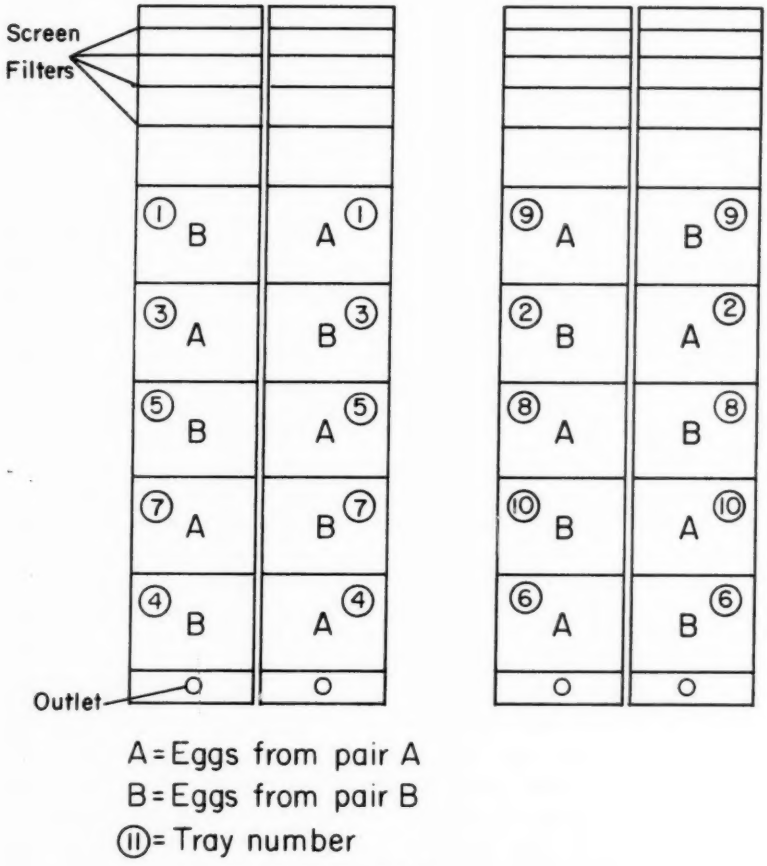

FIGURE 7

Diagrammatic arrangement of glass rod trays in cement troughs in experiment on effects of high intensity visible radiation on different developmental stages of the chinook salmon.

TABLE 8
DEVELOPMENT OF CHINOOK SALMON *Oncorhynchus tshawytscha*, IN RELATION TO TIME AND TEMPERATURE

Approximate percentage of completion of development (hatching = 100%)	Approximate number of temperature units	Description of developmental stage
0.8	9	Four cell stage.
2.7	25	Sixty-four cell stage.
11.0	101	Germ ring starts to form.
15.3	141	Germ ring covers ¼ of yolk surface, embryonic axis visible.
20.0	184	Blastoderm covers ½ yolk surface.
31.7	292	Brain, spinal cord, and mesodermal somites visible; optic vesicles forming.
36.3	334	Blastopore closed, optic vesicles well developed, eyes not pigmented, fins not formed.
47.9	441	Eyes pigmented, caudal fin starts to form, gill buds developing.
54.8	504	Nostrils and dorsal fin appear.
74.0	681	Cerebral hemispheres and ventral fins visible.
89.0	819	Dorsal and anal fin rays appear.
100	920	Fifty percent hatch.
172 to 185	1586 to 1706	Yolk-sac absorbed, fish are ready for first feeding.
310 to 414	2851 to 3808	Fingerlings weigh 2.4 to 4.2 grams and may be released in freshwater for seaward migration.
435	4000	Fingerlings weigh more than 4.3 grams and may be released directly into saltwater.

every three weeks. Fish were not fed on the day preceding measuring but were nonetheless exposed to light for two 20 minute periods. Weighing was accomplished by impounding the entire lot and discarding every other fingerling until the desired total of 50 was attained. The net containing the impounded fish was drained for 20 seconds prior to depositing the group in a counterbalanced pan of water. Group weights were recorded to the nearest gram on a Ohaus beam balance. The distance between snout and end of the scaled region was determined for each fish. This process was accomplished with a curved glass tube fastened with beeswax to a metal funnel. Salmon were measured through the glass tube with dividers. At the end of the study,

mean length of each group was compared to all other groups by the Student's t test[7] (Chapman, 1955).

Results

Effects of high-intensity visible radiation on different developmental stages of chinook salmon through the 92nd day of the study are summarized in Tables 9 and 10 and Figures and 8 and 9.

Results show that all zygotes irradiated during the first or second week of development, with one exception, die prior to hatching; moreover, few light-exposed embryos survive to the eyed stage. The exception was group 1A (Table 9) which produced 12 normal-appearing larvae or 2.3 percent of the total sample. However, irradiated fish differed from controls by hatching 2-3 days earlier, and dying shortly after hatching.

Embryos exposed during the third week of incubation suffered mortality of 74.4 (Pair A) and 100 (Pair B) per cent by day 92, with practically all mortality confined to the post-hatch period. Comparatively high premature hatching mortality was evident in group 3B but not in 3A. Accelerated hatch was observed in 3A and 3B, but larvae appeared normal in all respects. Many larvae which eventually died showed slower yolk-sac absorption than controls. About half the fish from group 3A that died during the fry stage were edematous.

Embryos irradiated during the fourth week of development were apparently unaffected at hatching. However, late larval and early fry stages experienced relatively high mortality and by day 92 survival was 4 to 10 percent lower than controls.

No differences were discerned by day 92 between controls and

[7]

$$t = \frac{\bar{X} - \bar{Y}}{Sp\sqrt{\frac{1}{N_x} + \frac{1}{N_y}}}$$

where $\bar{X}$, $\bar{Y}$ = sample means

$$Sp^2 = \text{pooled variance} = \frac{(N_x - 1)(S_x^2) + (N_y - 1)(S_y^2)}{N_x + N_y - 2}$$

S_x^2, S_y^2 = variances of respective samples

N_x, N_y = sample sizes

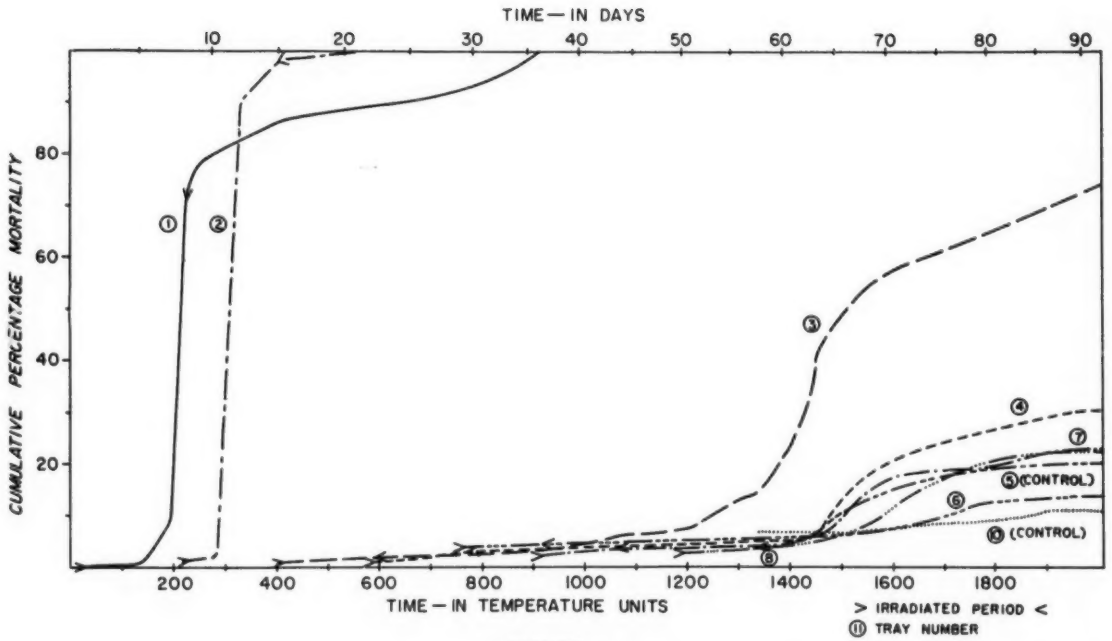

FIGURE 8

Observed mortality of Pair A chinook salmon to day 92. Irradiated groups were exposed to 157 foot-candles of fluorescent light for a period of seven days.

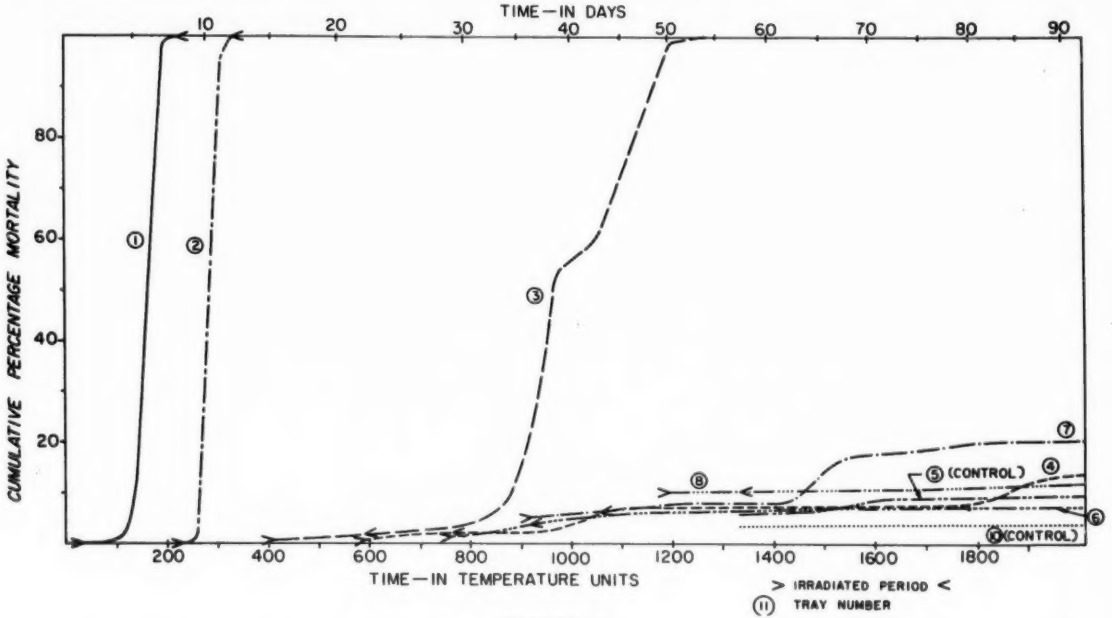

FIGURE 9

Observed mortality of Pair B chinook salmon to day 92. Irradiated groups were exposed to 157 foot-candles of fluorescent light for a period of seven days.

groups irradiated during the fifth week of incubation (Groups 6A and 6B).

Lots exposed to light during hatching processes (groups 7A and 7B) displayed up to 13 percent greater mortality than the controls. Most deaths occurred during the middle of the yolk-sac absorption stage and dead larvae exhibited larger yolk-sacs than live control larvae of the same age.

Larvae irradiated during the eighth week of development, when compared to the controls, were not affected by day 92.

Group 9A and 9B died shortly after hatching and prior to irradiation. The cause of the mortality is unknown but is probably associated with the large amount of silt deposited in troughs during the hatching processes. These compartments (see Fig. 7) are located near the inlet to the unfiltered lake water and consequently accumulated the most sediment. Data regarding these groups are considered acceptable until hatching, but are unacceptable after that time.

During the course of the study, no monsters (*i.e.* multiple structures, twinning, scoliosis, lordosis) were observed.

Data regarding parr mark distribution of chinook salmon fingerlings sacrificed at age 92 days are presented in Table 11. Analysis of mean number of parr marks of Pair A fingerlings by the *t* test indicates that no significant difference exists among groups. Comparison of mean parr mark number among Pair B salmon reveals, with one exception, no difference. The exception, fingerlings exposed to light during hatching processes, had fewer ($t = 2.05^*$) parr marks than the controls. This same group exhibited a mortality 13 percent higher than the controls, suggesting that reduction in parr mark number may be associated with decreasing survival. Variation in parr mark number among fingerlings from different brood lots, however, is statistically significant ($t = 12.34^{**}$) demonstrating the value of maintaining suitable control groups.

Observations on mortality and growth of salmon fingerlings between age 92 and 197 days are summarized in Tables 12 and 13. With one exception, mortality during the 105 day period did not exceed 4 per cent in any group. Group 3A (Table 12), however, suffered 38 per cent mortality during this period. Of the 47 dead fingerlings from group 3A, 18 had greatly distended abdomens. Post-mortem examination of these fish showed an accumulation of serous fluid in the abdominal cavity,

TABLE 9

SUMMARY OF OBSERVATIONS TO DAY 92, PAIR A CHINOOK SALMON

Irradiated groups were exposed to 157 foot-candles of fluorescent light for a period of seven days. Developmental stages are described in the text

Group	1	2	3	4	5	6	7	8	9	10
Number of eggs in sample	523	553	576	601	607	491	544	585	502	579
Irradiation period										
in days	1-8	8-15	15-22	22-29	control	29-36	36-43	50-57	dis-carded	con-trol
in temperature units	27-223	223-409	409-591	591-765		765-916	916-1058	1195-1332		
Percentage mortality by stages										
Stage I	87.4	99.0	1.1	0.9	1.3	1.4	0.3	0.7	0.0	2.4
Stage II	9.7	1.0	1.7	1.6	1.2	1.7	0.6	0.8	2.8	1.6
Stage III	0.6	—	0.3	0.5	0.0	0.6	0.6	0.2	0.6	0.7
Percentage successful hatch	2.3	0.0	96.9	97.0	97.5	96.3	98.5	98.3	96.6	95.3
Number of temperature units absorbed before 50 per cent of survivors hatch	874	—	865	880	895	885	895	890	895	885
. . . 95 per cent hatch	885	—	905	930	936	916	936	916	936	916

TABLE 9 (*Continued*)

Group	1	2	3	4	5	6	7	8	9	10
Number of eggs in sample	523	553	576	601	607	491	544	585	502	579
Percentage mortality by stages										
Stage IV	2.3	—	54.3	18.0	12.8	4.1	16.3	11.5	96.6	2.6
Stage V	—	—	17.0	9.1	4.6	5.7	4.8	9.0	—	3.5
Total percentage mortality by day 92	100.0	100.0	74.4	30.1	19.9	13.5	22.6	22.2	100.0	10.8

TABLE 10

SUMMARY OF OBSERVATIONS TO DAY 92, PAIR B CHINOOK SALMON

Irradiated groups were exposed to 157 foot-candles of fluorescent light for a period of seven days. For description of developmental stages, see text.

Group	1	2	3	4	5	6	7	8	9	10
Number of eggs in sample	496	421	481	510	481	528	508	480	453	434
Irradiation period										
in days	1-8	8-15	15-22	22-29	con-trol	29-36	36-43	50-57	dis-carded	con-trol
in temperature units	27-223	223-409	409-591	591-765		765-916	916-1058	1195-1332		
Percentage mortality by stages										
Stage I	100	100	1.2	1.3	3.0	1.9	3.7	3.3	1.0	0.8
Stage II	—	—	1.7	1.0	1.8	0.2	2.0	4.6	5.2	1.5
Stage III	—	—	2.5	0.8	0.0	0.9	0.2	0.2	0.4	0.2
Percentage successful hatch	0.0	0.0	94.6	96.9	95.2	97.0	94.1	91.9	93.4	97.5
Number of temperature units absorbed before 50 per cent of survivors hatch	—	—	853	880	900	880	900	900	900	900
. . . 95 per cent hatch	—	—	916	936	955	936	936	955	955	955
Percentage mortality by stages										
Stage IV	—	—	94.6	4.6	4.5	4.1	12.3	2.9	93.4	0.9
Stage V	—	—	—	5.7	0.2	0.4	2.6	0.8	—	0.4
Total percentage mortality by day 92	100.0	100.0	100.0	13.4	9.5	7.5	20.8	11.8	100.0	3.8

TABLE 11
PARR MARK FREQUENCY DISTRIBUTION ON THE LEFT SIDE OF PAIR A AND PAIR B CHINOOK SALMON FINGERLINGS SACRIFICED AT AGE 92 DAYS

Number of parr marks	Group						
	3A	4A	5A	6A	7A	8A	10A
8	1	3		1		1	
9	7	7	9	6	8	5	7
10	7	9	7	10	11	11	9
11	9	4	8	6	6	7	8
12	1	2	2	2		1	1
Average	10.08	9.80	10.11	10.08	9.92	10.08	10.12
Standard deviation	0.99	1.11	1.05	0.99	0.75	0.96	0.88

Number of parr marks	Group					
	4B	5B	6B	7B	8B	10B
7	1	2	3	5	2	2
8	11	6	5	10	10	11
9	8	9	10	7	8	7
10	3	4	5	3	3	5
11	1	4	2		2	
12	1					
Average	8.80	9.08	8.92	8.32	8.72	8.60
Standard deviation	1.12	1.18	1.16	0.95	1.06	0.91

a distended gut, and a grossly abnormal liver. Livers were pale yellow in color, enlarged, and contained an unusually high percentage of fat globules; further, fat globules were not of the shape and coloration usually found in normal liver tissue. This syndrome is characteristic of abnormal fat metabolism and could be attributable to pancreas damage (Donaldson, 1943). A cursory examination of the remainder of the dead fingerlings from group 3A showed no evidence of liver or visceral damage, and cause of death is unknown.

Statistical analysis of mean lengths of salmon at age 197 days are presented in Tables 14 and 15. Compared to controls, reduced growth was evident in groups exposed to light during the third, fourth, and sixth week of incubation. Mean lengths of larvae irradiated during the eighth week of development did not differ significantly from the controls. Apparently inconsistent results were obtained with groups subjected to light during the fifth week of development (groups 6A and 6B). Pair A fish were not affected but Pair B fish grew more

TABLE 12
SUMMARY OF OBSERVATIONS REGARDING LENGTH, WEIGHT, AND MORTALITY OF PAIR A CHINOOK SALMON FINGERLINGS BETWEEN AGE 92 AND 197 DAYS
Initial number in each group: 123

Day		Group 3	4	5	6	7	8	10
	Mean length	32.42	34.74	35.34	36.32	35.62	35.82	36.48
92	S.D.[8] length	1.39	1.44	1.20	2.07	1.25	1.61	1.34
	Mean weight	0.46	0.52	0.56	0.62	0.58	0.54	0.60
	Mean length	34.36	39.80	40.42	42.70	40.38	40.94	42.16
113	S.D. length	2.33	2.85	2.81	3.00	2.73	2.78	2.71
	Mean weight	0.56	0.86	0.94	1.06	0.92	0.98	1.00
	Mortality	6	1	2	1	2	3	3
	Mean length	39.82	47.04	48.32	49.24	46.26	47.76	49.08
134	S.D. length	3.67	3.82	2.33	2.80	4.12	3.05	3.37
	Mean weight	0.98	1.50	1.60	1.64	1.52	1.60	1.70
	Mortality	7	0	2	2	0	0	0
	Mean length	47.90	52.02	55.94	56.38	52.52	55.78	56.22
155	S.D. length	4.00	3.40	2.80	2.96	3.90	3.09	4.71
	Mean weight	1.56	2.04	2.36	2.48	2.12	2.38	2.38
	Mortality	18	0	0	2	1	0	0
	Mean length	54.30	57.44	61.56	62.00	59.58	61.98	62.62
176	S.D. length	4.36	4.18	3.07	3.75	3.28	3.02	3.43
	Mean weight	2.26	2.68	3.22	3.32	2.92	3.24	3.44
	Mortality	9	0	0	0	0	0	0
	Mean length	61.18	63.30	68.44	68.66	65.08	68.30	68.30
197	S.D. length	3.19	3.73	3.99	4.01	4.39	3.30	3.24
	Mean weight	3.24	3.62	4.12	4.28	3.76	4.20	4.24
	Mortality	7	0	1	0	0	0	0
	Total mortality	47	1	5	5	3	3	4

[8] S.D. = Standard deviation.

slowly than controls. The results, however, can be reconciled if one considers that Pair A is more resistant to light as evidenced by partial survival of group 3.

This study demonstrates that various developmental stages of chinook salmon react differently when exposed to 157 ft.-c. for a continuous period of seven days. In general, groups irradiated prior to onset of pigmentation exhibit high mortality, arrested state of development at death, accelerated hatch, reduced fingerling growth, and severe liver damage. Groups exposed to light during the remainder of embryonic development and the period encompassing hatching proc-

esses are less susceptible to visible rays but still display increased mortality, reduced growth, or both. Irradiation of larvae produced no apparent detrimental effects.

TABLE 13

SUMMARY OF OBSERVATIONS REGARDING LENGTH, WEIGHT, AND MORTALITY OF PAIR B CHINOOK SALMON FINGERLINGS BETWEEN AGE 92 AND 197 DAYS

Initial number in each group: 123

Day		Group 4	5	6	7	8	10
	Mean length	33.90	35.30	36.54	34.50	36.22	35.80
92	S.D. length	0.74	0.84	1.47	0.95	1.45	1.29
	Mean weight	0.44	0.52	0.60	0.50	0.58	0.56
	Mean length	37.34	40.20	42.20	36.62	41.96	41.26
113	S.D. length	3.21	1.83	2.45	2.05	3.02	2.38
	Mean weight	0.72	0.92	1.04	0.76	0.98	0.98
	Mortality	0	1	3	0	0	0
	Mean length	43.62	47.04	49.22	44.78	49.66	47.44
134	S.D. length	4.07	2.82	2.35	3.35	2.83	4.18
	Mean weight	1.28	1.58	1.68	1.36	1.72	1.56
	Mortality	1	0	0	0	0	0
	Mean length	50.40	55.48	56.84	51.40	55.98	55.74
155	S.D. length	4.58	2.39	3.06	2.86	2.58	3.32
	Mean weight	1.82	2.32	2.48	1.98	2.34	2.36
	Mortality	1	0	2	1	1	0
	Mean length	56.90	61.82	61.56	58.82	61.12	62.52
176	S.D. length	4.41	2.62	2.84	2.73	3.57	3.85
	Mean weight	2.60	3.28	3.24	2.90	3.20	3.40
	Mortality	0	1	0	1	0	1
	Mean length	64.84	68.86	67.40	65.38	68.38	69.84
197	S.D. length	6.31	4.30	4.09	3.73	3.10	3.53
	Mean weight	3.78	4.30	4.12	3.90	4.26	4.32
	Mortality	0	0	0	0	0	0
	Total mortality	2	2	5	2	1	1

TABLE 14

t-TEST ANALYSIS OF MEAN LENGTHS OF PAIR A SALMON EXPOSED TO 157 FOOT-CANDLES OF FLUORESCENT LIGHT AT DIFFERENT DEVELOPMENTAL STAGES

Group	Irradiated period (in days)	Mean length (in mm) at day 197	Group 3	4	6	7	8
5 and 10	controls	68.37	10.06**	7.36**	0.41	4.53**	0.10
3	15 to 22	61.18	×	2.67**	9.12**	4.55**	8.38**
4	22 to 29	63.30	×	×	6.93**	2.19*	6.21**
6	29 to 36	68.66	×	×	×	4.26**	0.43
7	36 to 43	65.08	×	×	×	×	3.71**
8	50 to 57	68.30	×	×	×	×	×

* Significant at the 0.05 level; ** significant at the 0.01 level.

TABLE 15
t-TEST ANALYSIS OF MEAN LENGTHS OF PAIR B SALMON EXPOSED TO 157 FOOT-CANDLES OF FLUORESCENT LIGHT AT DIFFERENT DEVELOPMENTAL STAGES

Group	Irradiated period (in days)	Mean length (in mm) at day 197	Group 4	6	7	8
5 and 10	controls	69.35	5.31**	2.79**	5.84**	1.50
4	22 to 29	64.84	×	2.41*	0.65	3.56**
6	29 to 36	67.40	×	×	2.58*	1.35
7	36 to 43	65.38	×	×	×	4.37**
8	50 to 57	68.38	×	×	×	×

* Significant at the 0.05 level; ** significant at the 0.01 level.

EXPERIMENT II: EFFECTS OF GRADED INTENSITIES OF VISIBLE RADIATION ON DIFFERENT EMBRYONIC STAGES OF CHINOOK SALMON

Methods

Salmon used in this portion of the study were part of a group returning to the University of Washington College of Fisheries via the University fish ladder. On Nov. 9, 1959 eggs from two females (Pair 1 and Pair 2) were each fertilized with milt from a single male and distributed as shown in Fig 10. After 24 hours, opaque eggs were removed and discarded. Remaining zygotes were exposed to different intensities of light at different developmental stages for a period of 120 hours as indicated by the latin square design (Snedecor, 1956) of Fig. 10. Growth and development of chinook salmon embryos are described in Table 8 and may be correlated with time of exposure to light. The physical characteristics of the parent fish used in Experiment II as well as those of Experiment I are listed in Table 16. The relationship be-

TABLE 16
PHYSICAL CHARACTERISTICS OF CHINOOK SALMON USED IN EXPERIMENTS I AND II

	Fork length (cm) male	female	Weight (kg) male	female	Total number of eggs produced	Egg diameter (mm)
Experiment I						
Pair A	86	94	—	—	5561	8.5
Pair B	89	83	—	—	4792	8.2
Experiment II						
Pair 1	84	87	7.8	11.2	4864	8.7
Pair 2	86	78	8.8	6.5	4268	8.1

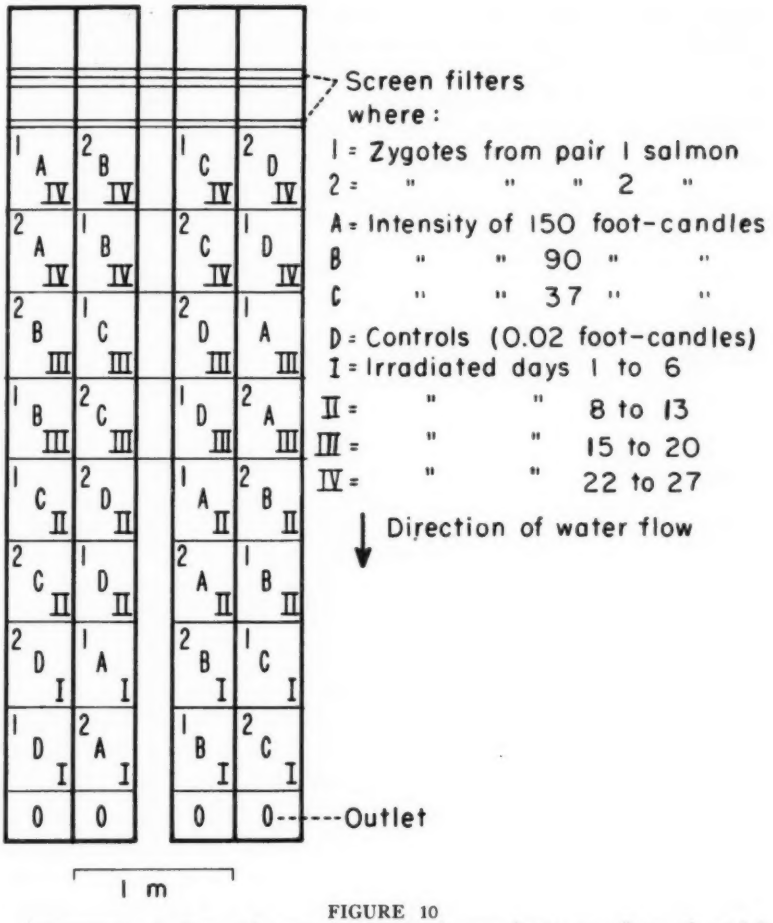
Screen filters
where :
1 = Zygotes from pair 1 salmon
2 = " " " 2 "
A = Intensity of 150 foot-candles
B " " 90 " "
C " " 37 " "
D = Controls (0.02 foot-candles)
I = Irradiated days 1 to 6
II = " " 8 to 13
III = " " 15 to 20
IV = " " 22 to 27
Direction of water flow
Outlet
1 m

FIGURE 10
Arrangement of glass rod trays in troughs in experiment on effects of graded intensities of visible radiation on four embryonic stages of the chinook salmon.

tween temperature units and average weekly water temperature during Experiment II is shown as Fig. 11; the same relationship being observed during Experiment I.

Groups were inspected at the beginning and end of each radiation

period (days 1, 6, 8, 13, 15, 20, 22, and 27). At this time opaque eggs were removed, preserved, and identified as to stage of development at death. After the 27th day, lots were examined every 48 hours until larvae had absorbed their yolk-sacs. The amount of light that non-irradiated groups recieved did not exceed 0.02 foot-candles. During

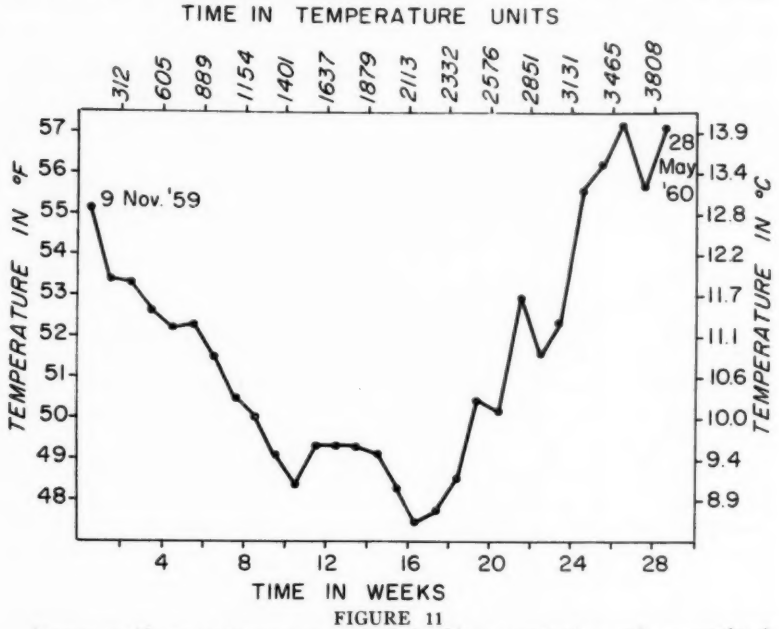

FIGURE 11

Average weekly water temperature and relationship to temperature units accumulated in experiment on effects of graded intensities of light on salmon.

inspection, lots received about 6 foot-candles of fluorescent light for 2 to 3 minutes. Methods employed in Experiment I regarding feeding, weighing, and measuring of fingerlings were again used in this experiment, but growth of fingerlings was determined at 25 day intervals rather than 21 days.

At age 200 days, about 30 fish from each group were randomly selected (every third fish until the desired total was attained), and preserved in three percent formalin. The remainder of the fingerlings, which normally migrate seaward at this phase of their development,

were released into Lake Washington. Three months after preservation all salmon were photographed and, using the method outlined by Bonham and Bayliff (1953), radiographed.

Radiographs showed the number of dorsal fin rays, anal fin rays, vertebrae, location and number of fused vertebrae, head length and fork length of individual fish. Procedures employed for enumeration of vertebrae and dorsal and anal fin rays were adapted from Seymour (1959). All radiographs were coded so that the actual group analyzed was unknown until all data were gathered. Photographing groups prior to radiography made possible individual enumeration of the vertical pigmented areas (parr marks) characteristic of juvenile chinook salmon. The *t*-test was employed to determine if illuminated groups were significantly different from controls regarding growth and meristic characters.

Results

Effects of graded intensities of visible radiation on four different embryonic stages of chinook salmon through day 100 are summarized in Tables 17 and 18 and Figures 12 and 13. It is obvious that salmon in the anterior portion of troughs were destroyed shortly after hatching (Tables 17, 18, Figs. 10, 12, 13). Inspection of these troughs showed large numbers of *Hydra oligactis*, presumably introduced into the system with the water. Subsequent experiments have indicated that hydras can kill recently-hatched salmon but have no effect on embryonic and late larval stages of fish (Eisler and Simon, 1961). Accordingly, data are invalid after hatching for all groups irradiated during the 22nd to 27th day and for the group exposed to 90 foot-candles (Pair 2) during the 15th to 20th day.

Analysis of percentage hatch, transformed by the arc-sine transformation, shows significant differences between developmental states, light intensities, and fish pairs (Table 19). Zygotes irradiated during age 1 to 6 days had lowest over-all survival by hatching. Increased hatch was exhibited by groups irradiated between age 8 to 13 days, 15 to 20 days, and 22 to 27 days in that order. However, drastically reduced hatch occurred in groups irradiated prior to the fifteenth day compared to groups irradiated after that period. Lots exposed to light of 150 ft.-c. produced the smallest hatch, 90 foot-candles a larger hatch, 37 ft.-c. a still larger hatch, and groups reared in near-darkness of

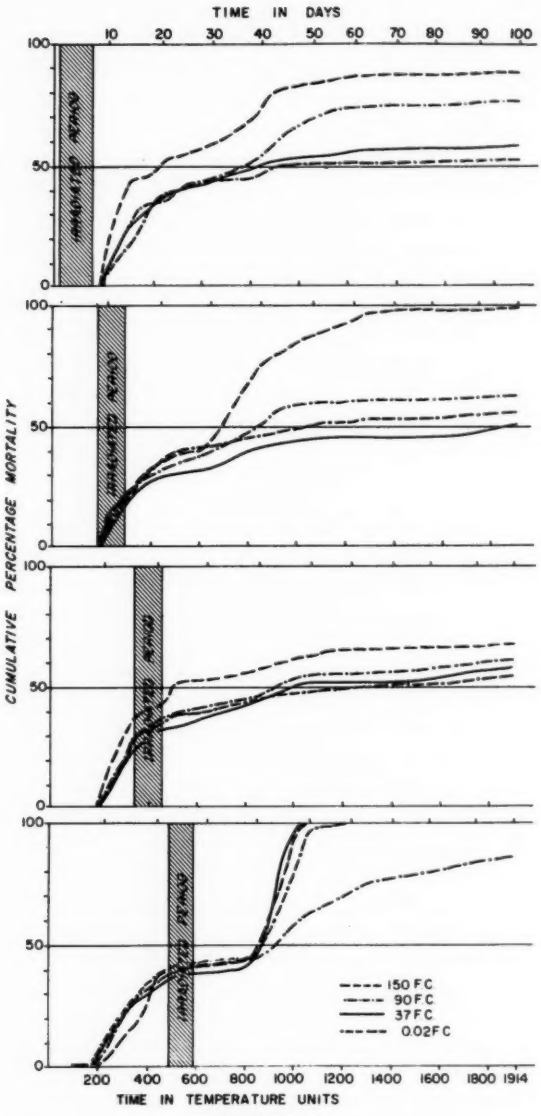

FIGURE 12

Cumulative percentage mortality of Pair 1 chinook salmon to day 100 in experiment on effects of graded intensities of light on different embryonic stages.

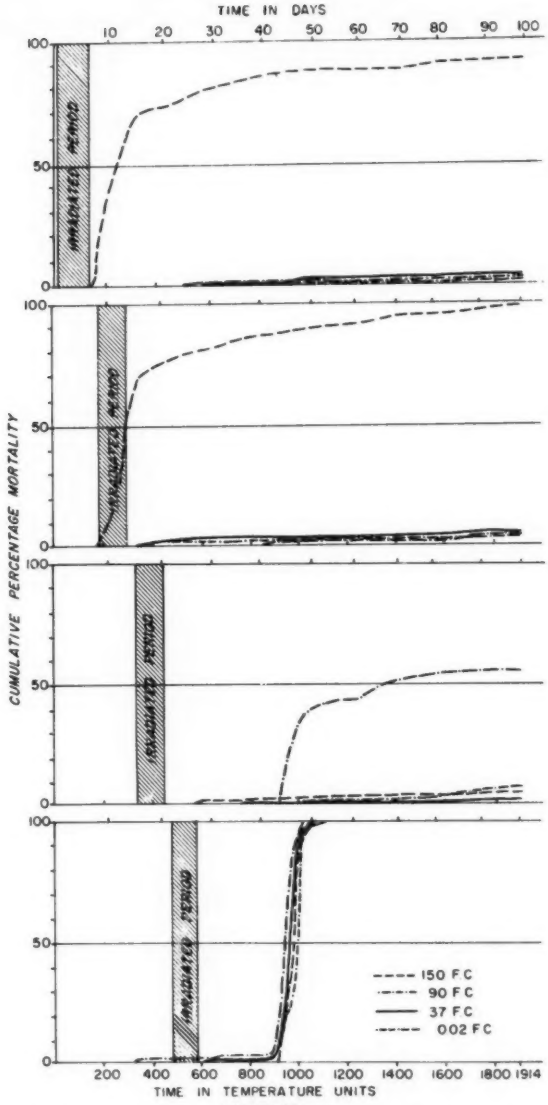

FIGURE 13

Cumulative percentage mortality of Pair 2 chinook salmon to day 100 in experiment on effects of graded intensities of light on different embryonic stages.

TABLE 17

SUMMARY OF OBSERVATIONS TO DAY 100, PAIR 1 CHINOOK SALMON

For description of developmental stages, see text.

Number of eggs in sample	187	193	196	195	192	201	188	202	200	186	198	197	186	188	188	200
Irradiation period in days	one to six				eight to thirteen				fifteen to twenty				twenty-two to twenty-seven			
in temperature units	24.6 to 141.4				183.8 to 291.6				333.6 to 440.9				483.3 to 585.9			
Light intensity (foot-candles)	150	90	37	0.02	150	90	37	0.02	150	90	37	0.02	150	90	37	0.02
Percentage mortality by stages																
Stage I	56.7	47.7	44.4	41.0	39.0	40.3	33.5	41.1	50.5	44.1	37.6	42.1	37.6	39.4	36.7	43.5
Stage II	10.7	11.9	5.1	7.2	14.1	10.4	5.3	8.4	6.5	3.8	3.2	3.5	7.4	3.7	4.2	5.0
Stage III	16.6	12.4	6.1	2.1	32.3	9.0	6.4	3.0	4.0	4.3	3.2	3.6	5.9	3.7	4.3	3.0
Percentage successful hatch	16.0	28.0	44.4	49.7	14.6	40.3	54.8	47.5	39.0	47.8	56.0	50.8	48.9	53.2	54.8	48.5
Number of temperature units absorbed before 50 per cent of survivors hatch	911	914	939	929	930	937	938	926	940	948	935	959	944	940	950	942
Percentage mortality by stages																
Stage IV	1.6	2.1	1.0	1.0	13.6	0.5	2.7	1.0	3.5	4.8	8.4	1.6	48.9	33.0	54.8	48.5
Stage V	2.1	2.6	1.1	1.0	0.5	3.0	2.6	3.4	3.0	3.2	5.3	4.0	—	7.4	—	—
Total percentage mortality by day 100	87.7	76.7	57.7	52.3	99.5	63.2	50.5	56.9	67.5	60.2	57.7	54.8	100	87.2	100	100

TABLE 18

SUMMARY OF OBSERVATIONS TO DAY 100, PAIR 2 CHINOOK SALMON

Developmental stages are described in the text.

Number of eggs in sample	198	204	202	206	188	202	190	209	208	203	198	186	199	204	202	200
Irradiation period in days	one to six				eight to thirteen				fifteen to twenty				twenty-two to twenty-seven			
in temperature units	24.6 to 141.4				183.8 to 291.6				333.6 to 440.9				483.3 to 585.9			
Light intensity (foot-candles)	150	90	37	0.02	150	90	37	0.02	150	90	37	0.02	150	90	37	0.02
Percentage mortality by stages																
Stage I	75.8	2.0	2.0	1.5	78.7	0.5	2.6	1.4	1.9	0.5	0.0	0.0	0.0	2.0	1.0	0.5
Stage II	8.6	0.0	0.5	0.0	6.9	0.5	0.0	1.0	0.0	0.0	0.5	0.0	0.0	1.0	0.0	0.0
Stage III	1.5	0.5	1.0	0.0	3.2	1.5	0.0	0.5	0.0	0.5	0.5	0.0	0.0	1.5	0.0	0.0
Percentage successful hatch	14.1	97.5	96.5	98.5	11.2	97.5	97.4	97.1	98.1	99.0	99.0	100	100	95.5	99.0	99.5
Number of temperature units absorbed before 50 per cent of survivors hatch	890	906	920	924	890	926	924	916	928	938	928	936	934	932	929	937
Percentage mortality by stages																
Stage IV	3.5	0.4	0.0	0.4	5.9	0.5	1.6	0.0	2.4	53.7	1.0	4.3	100	95.5	99.0	99.5
Stage V	1.5	0.0	0.5	1.0	3.2	0.5	0.0	0.4	0.5	1.0	0.5	2.7	—	—	—	—
Total percentage mortality by day 100	90.9	2.9	4.0	2.9	97.9	3.5	4.2	3.3	4.8	55.7	2.5	7.0	100	100	100	100

0.02 ft.-c. produced greatest survival. Pair 1 fish suffered heavier mortality than Pair 2 fish at all developmental stages and light intensities.

TABLE 19
ANALYSIS OF VARIANCE TABLE WITH PERCENTAGE HATCH OF PAIR 1 AND PAIR 2 SALMON AS THE RANDOM VARIABLE

Source of variation	Sum of squares	Degrees of freedom	Mean square	F value
Troughs	862.57	3	287.53	1.97
Developmental stages	1,464.00	3	488.00	3.34*
Light intensities	2,563.66	3	854.55	5.85**
Fish pairs	9,464.82	1	9,464.82	64.76**
Interaction	1,371.06	6	228.51	2.02
Fish × troughs	310.26	3	103.42	0.98
Fish × developmental stages	261.30	3	87.10	0.83
Fish × light intensities	496.74	3	165.58	1.58
Fish × error	629.59	6	104.93	
Correction	108,086.92			
Total	125,510.92			
Error	1,697.89	15	113.19	
Pooled error	3,068.95	21	146.14	

* Significant at the 0.05 level; ** significant at the 0.01 level.

Other salient features of Tables 17 and 18 include high premature hatching mortality, accelerated hatch, and arrested state of development prior to death of groups exposed to intensities of 90 and 150 ft.-c. Of interest are the Pair 2 groups exposed to 150 ft.-c. during the first two developmental stages (Fig. 13, Table 18). The relatively high mortality by day 100 indicates that a threshold dose affecting survival exists between 90 and 150 ft.-c.

During the study, six grossly abnormal salmon were discovered, all progeny of Pair 1 fish. Pair 2 groups, by contrast, did not contain any deformities. Five malformed fish were in the group exposed to 90 ft.-c. between the 8th and 13th day of incubation; the remaining fingerling had received 150 ft.-c. between the 15th and 20th day of life. All deformities were restricted to the posterior portion of the spinal column. Significantly, caudal deformation was noted although embryos were irradiated before the appearance of the structure (Table 8). Photographs of deformed fish surviving at age 200 days are shown as Fig. 14.

To avoid overcrowding, groups containing Pair 2 fingerlings were

This fish was exposed to an intensity of 150 f.c. between days 15 and 20. The spinal column has a coil-like appearance.

These fish were exposed to an intensity of 90 f.c. between days 8 and 13. Two additional fish displaying spinal deformities died between age 100 and 200 days.

FIGURE 14

Photographs of grossly abnormal salmon surviving at age 200 days (XI).

reduced to a maximum of 88 fish at day 100. Pair 1 groups contained a comparatively small number of survivors and were therefore not reduced in numbers. Observations regarding length, weight, and mortality are summarized in Tables 20 and 21. These data suggest that groups exposed to 150 ft.-c. prior to age 15 days exhibit: retarded growth as early as age 125 days; proportionately greater mortality by age 200 days; and, an apparently normal length-weight relationship as evidenced by the condition factors ($100 \times$ Weight in grams/(Length in cm)^3). No differences in length, weight, and mortality are evident among other groups irradiated prior to age 15 days. Similarly, no differences exist among groups exposed to light during the 15th to 20th day of incubation prior to age 175 days. At that time, however, the 37 ft.-c. group of Pair 1 (Table 20) and the 37, 90, and 150 ft.-c. groups of Pair 2 (Table 21) commence faster growth in length and weight than controls. Analyses of mean lengths of fingerlings at age 200 days are presented in Table 22. Statistical results confirm observational data, namely, that embryos exposed to high intensities of visible radiation prior to blastopore-closure are smaller than controls at age 200 days. Conversely, embryos exposed between blastopore-closure and retinal pigmentation exhibit accelerated growth at age 200 days when compared to control groups.

Data on head length, fork length, parr marks, number of vertebrae, and number of dorsal and anal fin rays of fingerlings sacrificed at age 200 days are summarized and presented graphically as Figures 15 and 16.

Mathematical analysis shows that average number of vertebrae significantly increases with increasing light intensity in groups irradiated between age 1 and 6 days (Table 23). However, this relationship is not evident among groups irradiated after day six. For example, embryos exposed to dim light (37 ft.-c.) between age 8 and 13 days tend to have fewer vertebrae than groups reared in darkness or groups exposed to bright light. No difference in mean vertebrae number is evident among lots irradiated after closure of the blastopore. Table 24 summarizes observations on fish with fused vertebrae. Although meager, these data suggest that survivors of Pair 1 salmon contain a greater proportion of individuals with fused vertebrae than Pair 2 salmon; also, fish with multiple fusions appear in proportionately greater numbers in light-exposed groups than in controls.

TABLE 20

SUMMARY OF OBSERVATIONS ON PAIR 1 CHINOOK SALMON FINGERLINGS FROM DAY 100 TO 200

Irradiated period (in days)	1-6	1-6	1-6	1-6	8-13	8-13	8-13	8-13	15-20	15-20	15-20	15-20
Light intensity (foot-candles)	0.02	37	90	150	0.02	37	90	150	0.02	37	90	150
Number in group at day 100	91	83	46	23	90	93	76	1	93	80	73	65
Mean weight (day 100)	.648	.554	.565	.565	.589	.581	.500	<.500	.462	.550	.534	.477
Mean weight (day 125)	1.14	1.00	1.04	0.82	1.02	0.94	0.90	—	0.86	0.90	0.92	0.90
Mean length	43.78	43.02	43.13	40.50	42.62	42.02	42.50	—	41.06	41.58	41.72	41.38
S.D. length	1.71	1.94	1.72	1.89	2.16	1.99	1.75	—	2.18	1.81	2.11	2.18
Mortality	1	0	0	1	0	5	0	1	2	3	1	1
Mean weight (day 150)	1.98	1.80	1.76	1.29	1.62	1.58	1.64	—	1.46	1.46	1.40	1.50
Mean length	52.42	51.64	51.04	48.05	50.58	49.48	50.18	—	48.28	48 64	48.00	48.96
S.D. length	2.63	2.88	2.05	2.30	3.66	2.88	2.20	—	2.43	2.78	2.36	2.17
Mortality	0	0	0	1	1	0	0	—	0	1	0	0
Mean weight (day 175)	2.92	3.02	2.91	2.25	2.90	2.84	2 82	—	2.42	2.54	2.42	2.48
Mean length	61.92	60.46	60.89	55.65	60.10	60.50	60.08	—	58.16	59.80	57.66	57.96
S.D. length	3.30	3.04	2.29	3.53	3.55	2.79	2.69	—	3.68	4.37	2.82	3.05
Mortality	0	1	0	1	1	0	1	—	0	0	0	1
Mean weight (day 200)	4.96	4.74	4.85	3.50	4.58	4.96	4.68	—	4.12	4.66	4.10	4.00
Mean length	70.72	69.06	70.41	62.90	68.64	70.86	69.24	—	66.30	69 22	67.04	65.84
S.D. length	5.04	4.59	4.68	4.34	5.56	5.86	4.80	—	4.59	5.96	4.50	4.07
Mortality	0	0	0	0	1	1	0	—	0	1	0	0
Condition factor	1.40	1.44	1.39	1.41	1.42	1.39	1.41	—	1.41	1.41	1.36	1.40

TABLE 21

SUMMARY OF OBSERVATIONS ON PAIR 2 CHINOOK SALMON FINGERLINGS FROM DAY 100 TO 200

Irradiated period (in days)	1-6	1-6	1-6	1-6	8-13	8-13	8-13	8-13	15-20	15-20	15-20	15-20
Light intensity (foot-candles)	0.02	37	90	150	0.02	37	90	150	0.02	37	90	150
Number in group at day 100	88	88	88	19	88	88	88	4	88	88	88	88
Mean weight (day 100)	.582	.546	.580	.316	.589	.588	.510	.375	.550	.568	.580	.533
Mean weight (day 125)	1.10	1.16	1.14	1.00	0.90	1.10	1.02	0.75	0.94	1.10	0.88	1.00
Mean length	43.56	43.70	43.70	39.71	43.24	43.68	42.78	38.50	42.34	42.80	41.96	42.90
S.D. length	1.92	1.28	1.51	3.55	2.31	1.45	2.06	3.11	2.23	1.57	2.23	1.81
Mortality	1	0	0	3	0	0	0	0	0	1	1	0
Mean weight (day 150)	1.82	1.90	1.92	1.31	1.76	1.78	1.72	1.00	1.56	1.64	1.48	1.66
Mean length	51.46	51.94	51.78	46.63	51.14	51.12	50.04	45.25	49.04	50.08	48.64	49.76
S.D. length	2.76	2.36	2.20	4.49	2.29	1.62	2.23	3.86	2.18	2.30	2.13	2.46
Mortality	0	0	0	1	0	0	0	0	0	1	0	0
Mean weight (day 175)	2.90	3.00	3.02	2.50	2.88	2.88	2.96	2.50	2.48	2.64	2.48	2.80
Mean length	60.58	60.66	62.38	56.64	60.98	60.48	60.36	52.75	57.92	58.76	58.10	58.80
S.D length	3.59	3.07	3.28	4.97	3.35	2.53	3.42	5.32	4.08	2.88	2.99	2.63
Mortality	0	0	0	1	0	0	0	0	0	0	0	1
Mean weight (day 200)	4.22	4.28	4.44	3.21	4.34	4.50	4.30	3.25	3.70	4.18	3.96	3.98
Mean length	68.08	67.72	68.02	63.71	67.76	68.60	67.90	60.00	63.72	66.90	66.16	66.00
S.D. length	4.38	4.00	4.44	6.29	4.53	3.62	4.60	5.42	4.57	3.29	3.18	4.58
Mortality	1	0	0	0	1	0	0	0	1	0	0	0
Condition factor	1.34	1.38	1.41	1.24	1.40	1.39	1.37	1.50	1.43	1.40	1.37	1.38

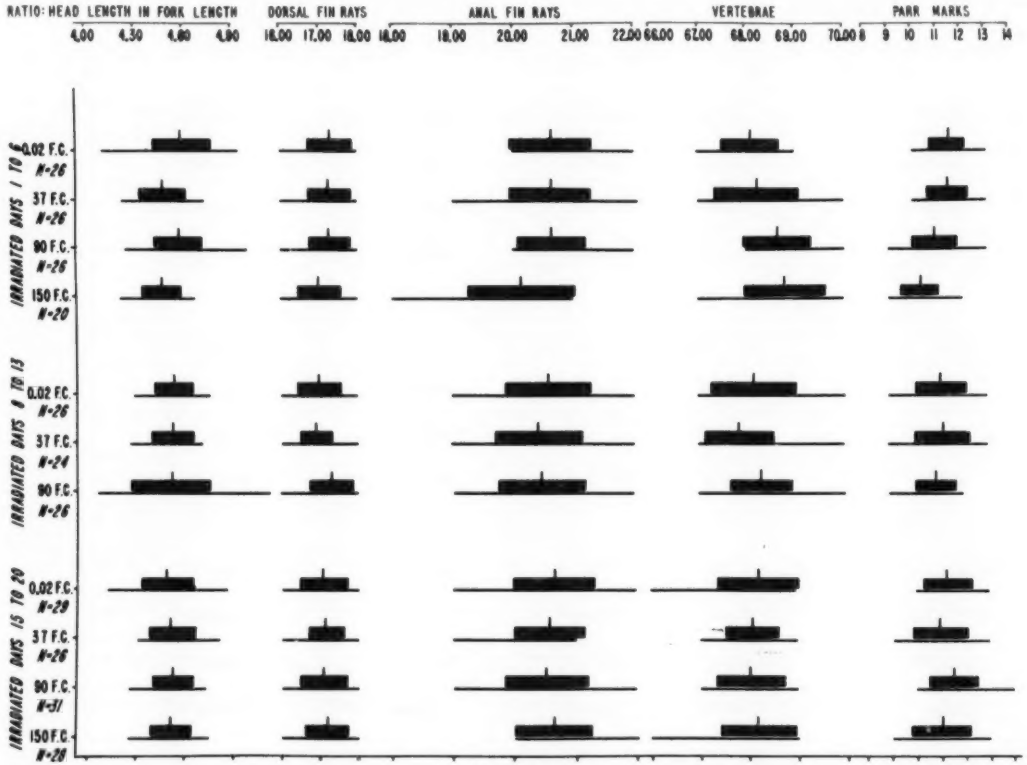

FIGURE 15

Graphical presentation of meristic and non-meristic characters of Pair 1 salmon exposed to different intensities of light at different developmental periods and sacrificed at age 200 days. The range is shown by the long horizontal line; the mean by the short vertical bar; one standard deviation by one-half of a black bar; and N is equal to the sample size.

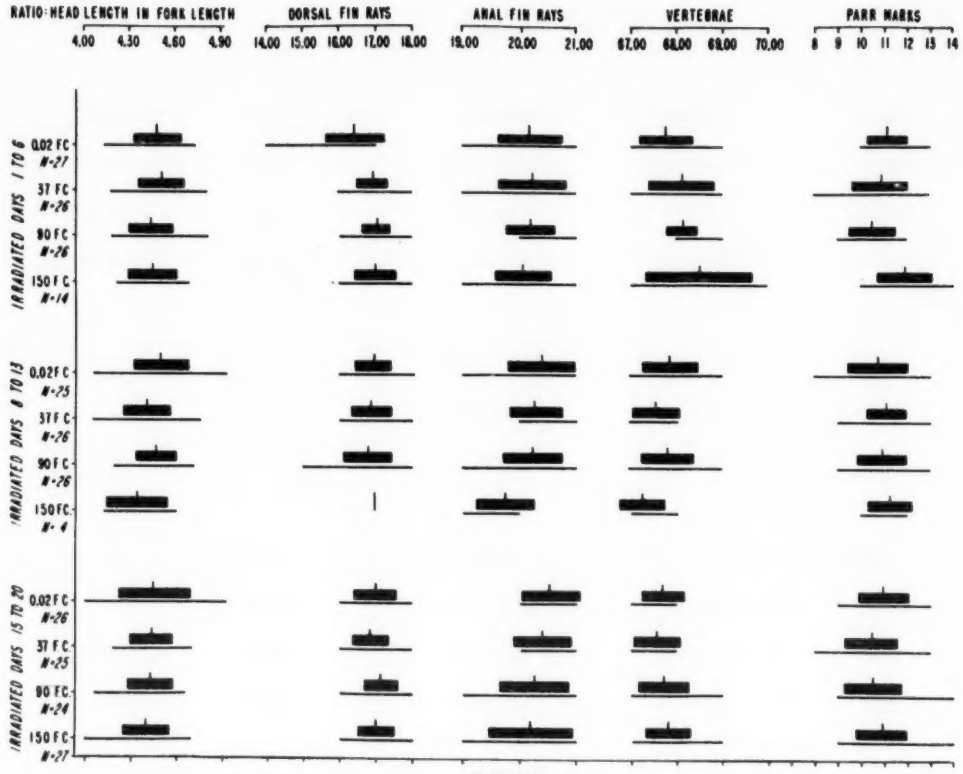

FIGURE 16

Graphical presentation of meristic and non-meristic characters of Pair 2 salmon exposed to different intensities of light at different developmental periods and sacrificed at age 200 days.

Groups irradiated during the first week of development, with one exception, show a significant decrease in parr mark number with increasing light intensity (Table 25). The aberrant (Pair 2) group had been exposed to the brightest illumination (150 ft.-c.) and also exhibited the highest number of parr marks. To explain this discrepancy,

TABLE 22
ANALYSIS OF MEAN LENGTH DIFFERENCES OF CHINOOK SALMON FINGERLINGS AT AGE 200 DAYS BY THE *t*-TEST

Light intensity (foot-candles)	Irradiated period (days)	Mean length at day 200 (mm)	Light intensity		
			37	90	150
		Pair 1			
0.02	1-6	70.72	1.72	0.31	6.09**
37	1-6	69.06	×	1.43	5.15**
90	1-6	70.41	×	×	6.12**
150	1-6	62.90	×	×	×
0.02	8-13	68.64	1.94	0.58	
37	8-13	70.86	×	1.51	
90	8-13	69.24	×	×	
0.02	15-20	66.30	2.75**	0.81	0.53
37	15-20	69.22	×	2.06*	3.31**
90	15-20	67.04	×	×	1.40
150	15-20	65.84	×	×	×
		Pair 2			
0.02	1-6	68.08	0.43	0.07	2.75**
37	1-6	67.72	×	0.36	2.71**
90	1-6	68.02	×	×	2.72**
150	1-6	63.71	×	×	×
0.02	8-13	67.76	1.02	0.15	3.25**
37	8-13	68.60	×	0.86	4.41**
90	8-13	67.90	×	×	3.27**
150	8-13	60.00	×	×	×
0.02	15-20	63.72	3.59**	2.78**	2.38*
37	15-20	66.90	×	0.87	1.01
90	15-20	66.16	×	×	0.18
150	15-20	66.00	×	×	×

* Significant at the 0.05 level; ** significant at the 0.01 level.

consider the high mortality of this exceptional group (Fig. 13) and the fact that harmful effects of radiation are drastically reduced with the advent of retinal pigmentation (Tables 7, 9-15, 17-23, 25). It seems reasonable to suggest that high light intensities will act as a selective lethal agent on that portion of the population with inadequate body pigmentation; thus, all results may be reconciled. No significant

trends regarding number of parr marks are evident among groups irradiated after the first week.

Number of anal fin rays decreases with increasing light intensity at all developmental stages but survivors of different brood lots re-

TABLE 23
ANALYSIS OF THE MEAN NUMBER OF VERTEBRAE OF CHINOOK SALMON FINGERLINGS AT AGE 200 DAYS BY THE *t*-TEST

Exposure period (in days)	Light intensity (foot-candles)	Mean	Light intensity		
			37	90	150
		Pair 1			
1-6	0.02	68.12	0.56	3.03**	3.27**
1-6	37	68.23	×	1.95	2.25*
1-6	90	68.65	×	×	0.65
1-6	150	68.80	×	×	×
8-13	0.02	68.15	1.42	0.73	
8-13	37	67.83	×	2.54*	
8-13	90	68.31	×	×	
15-20	0.02	68.21	0.68	0.89	0.12
15-20	37	68.08	×	0.26	0.50
15-20	90	68.03	×	×	0.71
15-20	150	68.18	×	×	×
		Pair 1			
1-6	0.02	67.78	1.90	2.61*	2.68*
1-6	37	68.12	×	0.00	1.30
1-6	90	68.12	×	×	1.59
1-6	150	68.50	×	×	×
8-13	0.02	67.88	2.20*	0.44	1.96
8-13	37	67.54	×	1.80	1.06
8-13	90	67.81	×	×	1.85
8-13	150	67.25	×	×	×
15-20	0.02	67.69	0.97	0.11	0.93
15-20	37	67.56	×	0.98	1.85
15-20	90	67.71	×	×	0.74
15-20	150	67.81	×	×	×

* Significant at the 0.05 level; ** significant at the 0.01 level.

act differently at various developmental stages (Table 26). For example, Pair 1 fish exposed to light of high intensity during age 1 to 6 days have significantly fewer anal fin rays than the controls. No trend is evident among Pair 1 lots irradiated after age 6 days. On the other hand, number of anal fin rays of Pair 2 lots is unaffected by light during the first week of development, but lots irradiated during age 8 to 13 days and during age 15 to 20 days display a reduction in number of anal fin rays with increasing light intensity.

Analysis of the ratio fork length/head length are presented in Table 27. Pair 1 salmon irradiated during the first to sixth day of development exhibit an inconsistent trend towards a proportionately larger head with increasing light intensities. This phenomenon was not noted among other lots; therefore, in the absence of supporting

TABLE 24
SUMMARY OF OBSERVATIONS ON CHINOOK SALMON WITH FUSED VERTEBRAE

Exposure period (in days)	Light intensity (foot-candles)	Number of fish with one or more fused vertebrae: (location of fused vertebrae)
	Pair 1 chinook salmon	
1-6	0.02	2: (19-20; 20-21) (50-51)
1-6	37	1: (19-20)
1-6	90	0
1-6	150	1: (14-15; 15-16; 16-17)
8-13	0.02	1: (12-13)
8-13	37	3: (16-17) (55-56; 59-60) (17-18)
8-13	90	2: (13-14) (39-40; 56-57)
15-20	0.02	3: (7-8) (16-17) (64-65)
15-20	37	1: (11-12)
15-20	90	1: (12-13; 13-14; 16-17)
15-20	150	4: (17-18) (16-17) (16-17) (64-65)
	Pair 2 chinook salmon	
1-6	0.02	0
1-6	37	1: (15-16)
1-6	90	1: (15-16)
1-6	150	0
8-13	0.02	0
8-13	37	0
8-13	90	0
8-13	150	0
15-20	0.02	0
15-20	37	3: (62-63) (13-14; 16-17) (63-64)
15-20	90	0
15-20	150	0

evidence, no positive statement is made regarding effects of light on ratio of head length to fork length.

Statistical treatment of dorsal fin ray data indicate that Pair 1 groups irradiated during the first week show decreasing number of dorsal fin rays with increasing light intensities; but, this trend is reversed with Pair 2 fish (Table 28). Since results are not consistent, no statement is made on this portion of the data. Of interest are Pair 1 embryos exposed to partial darkness (37 ft.-c.) during age 8 to

13 days. Resultant fingerlings had fewer dorsal fin rays than groups exposed to bright light or reared in darkness. Similar results are obtained with the Pair 2 group irradiated during age 15 to 20 days.

This experiment demonstrates that various embryonic stages of chinook salmon react differently when exposed to graded intensities of

TABLE 25
ANALYSIS OF THE MEAN NUMBER OF PARR MARKS OF CHINOOK SALMON FINGERLINGS AT AGE 200 DAYS BY THE *t*-TEST

Exposure period (in days)	Light intensity (foot-candles)	Mean	Light intensity		
			37	90	150
		Pair 1			
1-6	0.02	11.46	0.19	2.56*	5.45**
1-6	37	11.42	×	2.26*	4.86**
1-6	90	10.89	×	×	2.35*
1-6	150	10.30	×	×	×
8-13	0.02	11.15	0.18	1.06	
8-13	37	11.21	×	1.21	
8-13	90	10.89	×	×	
15-20	0.02	11.35	1.24	0.81	1.20
15-20	37	11.00	×	2.01*	0.00
15-20	90	11.55	×	×	1.96
15-20	150	11.00	×	×	×
		Pair 2			
1-6	0.02	11.15	0.93	2.51*	2.46*
1-6	37	10.89	×	1.19	2.70*
1-6	90	10.54	×	×	4.23**
1-6	150	11.93	×	×	×
8-13	0.02	10.72	1.30	0.62	0.79
8-13	37	11.12	×	0.72	0.29
8-13	90	10.92	×	×	0.58
8-13	150	11.25	×	×	×
15-20	0.02	10.96	1.70	1.28	0.25
15-20	37	10.44	×	0.33	1.52
15-20	90	10.54	×	×	1.09
15-20	150	10.89	×	×	×

* Significant at the 0.05 level; ** significant at the 0.01 level.

light (0.02 to 150 ft.-c.) for a continuous period of 120 hours. Zygotes irradiated at the 64 cell stage of development were particularly affected and exhibited measurable graded responses to graded intensities of light. Comparatively high light intensities (*i.e.* 150 or 90 ft.-c.) produced an arrested state of development prior to death, high premature hatching mortality, accelerated hatch, increased embryonic and larval mortality, reduced fingerling growth in length and weight, decrease in

mean number of anal fin rays, decrease in parr mark number, and an increase in mean vertebrae number of fingerlings. Embryos exposed to bright light between age 8 and 13 days, when compared to controls, displayed heavy mortality of embryos and larvae which was especially severe immediately prior to hatching, a reduction in fingerling growth,

TABLE 26
ANALYSIS OF THE MEAN NUMBER OF ANAL FIN RAYS OF CHINOOK SALMON FINGERLINGS AT AGE 200 DAYS BY THE *t*-TEST

Exposure period (in days)	Light intensity (foot-candles)	Mean	Light intensity		
			37	90	150
		Pair 1			
1-6	0.02	20.65	0.00	0.00	2.19*
1-6	37	20.65	×	0.00	2.19*
1-6	90	20.65	×	×	2.37*
1-6	150	20.15	×	×	×
8-13	0.02	20.58	0.80	0.59	
8-13	37	20.42	×	0.22	
8-13	90	20.46	×	×	
15-20	0.02	20.66	0.46	0.80	0.07
15-20	37	20.58	×	0.36	0.40
15-20	90	20.52	×	×	0.75
15-20	150	20.64	×	×	×
		Pair 2			
1-6	0.02	20.19	0.29	0.05	0.65
1-6	37	20.23	×	0.28	0.87
1-6	90	20.19	×	×	0.85
1-6	150	20.07	×	×	×
8-13	0.02	20.40	0.90	1.11	2.04
8-13	37	20.27	×	0.29	1.99
8-13	90	20.23	×	×	1.66
8-13	150	19.75	×	×	×
15-20	0.02	20.54	0.98	1.82	2.02*
15-20	37	20.40	×	0.95	1.22
15-20	90	20.25	×	×	0.34
15-20	150	20.19	×	×	×

* Significant at the 0.05 level.

increased incidence of spinally-deformed fish and a decrease in mean number of anal fin rays of resultant fingerlings. Groups exposed to 37 ft.-c. during age 8 to 13 days produced fingerlings with fewer vertebrae and fewer dorsal fin rays than groups reared in near-darkness of 0.02 ft.-c. or groups exposed to bright light. Salmon irradiated between blastopore-closure and retinal pigmentation (age 15 to 20 days) were relatively unaffected when compared to groups irradiated prior to age 15 days; however, these embryos when compared to controls exhibited

reduced hatch, accelerated fingerling growth in length and weight, and reduction in mean number of fingerling anal fin rays. Embryos exposed to 37 ft.-c. during age 15 to 20 days produced fingerlings with fewer dorsal fin rays than fish reared in near-darkness or exposed to 150 ft.-c. Embryos exposed to radiation at the onset of retinal

TABLE 27

ANALYSIS OF THE MEAN RATIO $\frac{\text{FORK LENGTH}}{\text{HEAD LENGTH}}$ OF CHINOOK SALMON FINGERLINGS AT AGE 200 DAYS BY THE *t*-TEST

Exposure period (in days)	Light intensity (foot-candles)	Mean	Light intensity		
			37	90	150
		Pair 1			
1-6	0.02	4.59	2.56*	0.43	2.61*
1-6	37	4.48	×	3.46**	0.17
1-6	90	4.57	×	×	2.54*
1-6	150	4.47	×	×	×
8-13	0.02	4.54	0.38	0.47	
8-13	37	4.53	×	0.22	
8-13	90	4.52	×	×	
15-20	0.02	4.50	0.70	0.97	0.48
15-20	37	4.52	×	0.19	0.30
15-20	90	4.53	×	×	0.55
15-20	150	4.51	×	×	×
		Pair 2			
1-6	0.02	4.48	0.75	1.11	0.63
1-6	37	4.51	×	1.86	1.23
1-6	90	4.44	×	×	0.26
1-6	150	4.45	×	×	×
8-13	0.02	4.50	1.98	0.61	1.67
8-13	37	4.41	×	1.65	0.80
8-13	90	4.47	×	×	1.80
8-13	150	4.34	×	×	×
15-20	0.02	4.45	0.28	0.46	0.96
15-20	37	4.44	×	0.26	0.93
15-20	90	4.43	×	×	0.63
15-20	150	4.40	×	×	×

* Significant at the 0.05 level; ** significant at the 0.01 level.

pigmentation (days 22 to 27) are apparently unaffected by light at time of hatching but information is not available after hatching.

DISCUSSION

Although electromagnetic energy influences growth and development of aquatic organisms, radiation effects may be modified by

temperature or chemical structure of the environment (Haggqvist and Hevesy, 1958; Gros *et al.*, 1959); moreover, factors which produce radiomimetic effects include chemicals (Hyman, 1921) and temperature (Hubbs, 1926; Seymour, 1959). Stockard (1921) states that changes in temperature, and oxygen supply are the most frequent

TABLE 28
ANALYSIS OF THE MEAN NUMBER OF DORSAL FIN RAYS OF CHINOOK SALMON FINGERLINGS AT AGE 200 DAYS BY THE *t*-TEST

Exposure period (in days)	Light intensity (foot-candles)	Mean	Light intensity		
			37	90	150
		Pair 1			
1-6	0.02	17.31	0.26	0.26	1.86
1-6	37	17.27	×	0.00	1.66
1-6	90	17.27	×	×	1.66
1-6	150	17.00	×	×	×
8-13	0.02	17.00	0.59	1.99	
8-13	37	16.92	×	2.84**	
8-13	90	17.31	×	×	
15-20	0.02	17.10	0.34	0.03	0.49
15-20	37	17.15	×	0.40	0.18
15-20	90	17.09	×	×	0.55
15-20	150	17.18	×	×	×
		Pair 2			
1-6	0.02	16.44	2.75**	3.48**	2.32*
1-6	37	16.92	×	1.13	0.51
1-6	90	17.04	×	×	0.35
1-6	150	17.00	×	×	×
8-13	0.02	16.92	0.31	0.93	0.32
8-13	37	16.89	×	0.71	0.44
8-13	90	16.77	×	×	0.70
8-13	150	17.00	×	×	×
15-20	0.02	17.00	1.09	0.86	0.00
15-20	37	16.84	×	2.16*	1.21
15-20	90	17.13	×	×	0.96
15-20	150	17.00	×	×	×

* Significant at the 0.05 level; ** significant at the 0.01 level.

causes of embryonic death and of monstrous development. He says that the primary action of all treatments is to retard the rate of development, and type of deformity that results depends simply upon the developmental moment at which interruption occurs. Stockard, however, has not considered the processes by which such changes take place. It is quite probable that different stimuli produce the same end result via unlike pathways.

Sparrow and Rubin (1951) review the energy transfer mechanisms by which radiations affect biological systems. They indicate that damaging effects of radio waves and infra-red radiations are thermal responses and rarely represent changes at a chemical level; but, energies in the region of visible light can affect chemical structure. Sparrow and Rubin describe the photochemical reaction as a process occurring in three rather indistinct stages. The radiation is first absorbed to form an "excited" molecule which then either decomposes or re-emits energy by either fluorescence, collision, or chemical reaction. The products of this second step may be reactive atoms or radicals which may cause secondary reactions such as molecular rearrangements, chain reactions, polymerization, or depolymerization. Although energies from the visible portion of the spectrum are not powerful enough to produce ionization, energy imparted to a molecule by ultra-violet light is sometimes sufficient to ionize it by completely ejecting an orbital electron. Ionization, aver Sparrow and Rubin, has generally been considered responsible for most of the radiobiological effect.

The present study shows that visible radiation can measurably affect salmon embryos and resultant fingerlings. The processes by which various physiological systems are modified may be attributed to thermal, photochemical, and photoelectric properties of the experimental light source. Thermal effects are indicated by accelerated hatch of embryos exposed to light during the earlier developmental stages. These results agree with the findings of others who report that premature hatch occurs after exposure to visible light (Page, 1885; Smith, 1916; Murisier, 1920; Dannevig, 1947; Anon., 1957; Hamdorf, 1960). Clark (1922) mentions that light energy absorbed by a body may increase molecular motion and result in a temperature rise.

Observed changes in mortality rates, meristic characters, gross histology, anatomy, and growth patterns are similar to the radiobiological damage pattern, and suggest that visible radiation has photoelectric properties. For example, death results when early embryonic stages of fish are exposed to high dosages of X-radiation (Welander *et al.*, 1948; Eisler, 1959), ultra-violet light (Haempel and Lechler, 1931; Bell and Hoar, 1950) or visible radiation (Lyubitskaya, 1956; Hamdorf, 1960). The present study shows that embryos exposed to illumination produce a comparatively high number of fingerlings which suffer from spinal and caudal deformities, organ damage, or edema. Similar results were

obtained with X-rays (Solberg, 1938a; Welander *et al.*, 1948) and ultra-violet light (Hinrichs, 1925; Hinrichs and Genther, 1931; Bell and Hoar, 1950). Delayed yolk-sac absorption was evident among resultant larvae when, as embryos, they were subjected to ultra-violet (Bell and Hoar, 1950) or visible light (Lindsey, 1958). As demonstrated in this study, reduction in number of fin rays occurs when embryos are exposed to light. Comparable results were observed on exposure to X-rays (Welander, 1954) or to visible light (Lindsey, 1958). Although visible radiation is theoretically incapable of producing ionizing effects (Sparrow and Rubin, 1951), energy from fluorescent light appears to have photoelectric properties. Clark (1922) suggests that light longer than 3000A acts on the living cell by ionizing photoelectric constituents in the presence of sensitizers. The sensitizing substances, perhaps secreted by the cell itself, may affect the surface condition of constituents so that their photoelectric threshold is shifted into the visible and ionization occurs upon illumination by visible or near ultra-violet light.

Investigations show that embryos subjected to bright light will produce fingerlings with fewer vertebrae than embryos reared under comparatively subdued lighting conditions (Dannevig, 1932b; McHugh, 1954; Lindsey, 1958). Vibert (1954), however, reports that embryos reared under conditions of partial darkness had more vertebrae than lots reared under higher or lower intensities. This study shows that zygotes exposed to increased light at the beginning of the 64-cell stage of development produce fish with an increased number of vertebrae. Embryos exposed to dim light of 37 ft.-c after the eighth day of incubation, however, hatched fish with fewer vertebrae than lots illuminated by 150 ft.-c. or by 0.02 ft.-c. A strict comparison between the present inquiry and other studies can not be made owing to large differences in duration and intensity of illumination, species, and developmental stage irradiated.

Since experiments were terminated after 200 days, no data are available on latent effects of high-intensity visible radiation such as sterility or reduced life span. Viability of sex products of embryos subjected to other components of the electromagnetic spectrum has not yet been documented, and promises to be an interesting field of investigation. At present, more research is needed to ascertain the roles played by enzymes, hormones, and other biochemical compounds after exposure to visible radiation.

Positive or negative phototaxis, depending on species and developmental stage, was noted among larval fish (Dannevig, 1932a; Dannevig and Sivertsen, 1933; Stuart, 1953; Hoar *et al.*, 1957; Woodhead, 1957). In the current investigation, salmon larvae reacted with erratic swimming movements when suddenly confronted with an intensity of about 9 ft.-c. of artificial light (from 0.02 ft.-c.). Locomotor disorganization displayed by larvae is analogous to swimming movements of small plectognath fishes exposed to bright light (Breder, 1942).

One finding of this study is the decrease in sensitivity to radiation with increasing differentiation of the embryo. Irradiation of early developmental stages by X-rays (Solberg, 1938a; Welander, 1954) and ultra-violet rays (Hinrichs, 1925) showed them to be more sensitive than older embryos. However, recently-fertilized eggs were more resistant than eyed stages when exposed to ultra-violet light (Haempel and Lechler, 1931). Hamdorf (1960) found that trout eggs exposed to visible light increased in sensitivity until the heart started action, after which time sensitivity rapidly decreased; but, Hamdorf exposed each stage to light repeatedly whereas the present study used single exposures. These differences may be a function of the metabolic rate of the dividing cell. Packard (1931) indicates that susceptibility to X-irradiation rises from prophase to telophase and then falls abruptly when division is completed. Results suggest that both degree of embryonic differentiation and metabolic rate of the dividing cell are factors influencing radiosensitivity.

Observations suggest that damage to salmon embryos by light might be related to degree of pigmentation. Groups irradiated prior to formation of retinal pigments show especially high mortality and severe alteration of organ systems and meristic characters. Lots exposed during formation of retinal and body pigments exhibit marked decrease in mortality and only slight modification of meristic characters. Little or no biological damage is evident among later embryonic stages which contain large amounts of pigments. Therefore, it is probable that melanophores help shield differentiating cells of salmon embryos from harmful effects of visible light. Results indicate that high intensities of visible radiation act as a selective lethal agent on that portion of the population with less protective pigments (*i.e.*, fewer parr marks). Thus, survivors of illuminated groups which experienced heavy mortality have a greater number of parr marks than the controls.

Lower intensities cause little mortality but light-exposed embryos exhibit fewer parr marks than the dark-reared group. Similar findings were obtained with X-rays (Welander *et al.*, 1948; Welander, 1954), ultra-violet radiation (Bell and Hoar, 1950), and visible light (Eisler, 1958).

Factors which can measurably alter effects of fluorescent light on chinook salmon eggs and larvae include intensity of light source, developmental stage illuminated, duration of exposure, and degree of embryonic pigmentation. Physiological effects of visible radiations on fish embryos are quite similar to those produced by temperature or ionizing radiations, and suggest that the experimental light source has thermal, photochemical, and photoelectric properties.

Summary

In a preliminary experiment, embryonic and larval developmental stages of rainbow trout were exposed for 72 hours to 157 foot-candles of fluorescent light characterized by wave length range of 3662 to about 7000 Angstrom units with mean energy transmission peak near 5600A (green-yellow portion of spectrum). Results indicate that embryonic stages irradiated prior to onset of extensive body pigmentation exhibit high mortality and arrested development prior to death; but, groups irradiated after that period are apparently unaffected by light.

Results of the first experiment with chinook salmon show that various developmental stages react differently when exposed for 168 hours to 157 ft.-c. of the previously-described light source. In general, embryos subjected to artificial light before formation of retinal pigments display high mortality at all stages of growth, retarded development at death, accelerated hatch, reduced fingerling growth, and severe liver damage. Groups irradiated during the remainder of embryonic development, including the period encompassing hatching processes, are less susceptible to visible rays but still exhibit increased mortality, reduced growth, or both. Exposure of larvae to fluorescent light produced no measurable changes by the end of the 197 day experiment. Large variations were evident among progeny of different brood lots regarding most experimental criteria.

For the final portion of the study, four embryonic stages of chinook salmon were exposed to different intensities of the experimental light

source for a continuous period of 120 hours. Zygotes subjected to radiation at the beginning of the 64 cell stage of development were especially affected and exhibited measurable graded responses to graded intensities of light; thus, comparatively high intensities (*i.e.*, 150 or 90 ft.-c.) produced arrested development at time of death, high premature hatching mortality, accelerated hatch, increased embryonic and larval mortality, reduced fingerling growth in length and weight, decrease in mean number of anal fin rays and parr marks of resultant fingerlings, and increase in mean vertebrae number. Embryos exposed to light of high intensity between age 8 and 13 days, when compared to controls, displayed high mortality which was especially severe immediately prior to hatching, reduced fingerling growth, a large number of spinally-deformed fingerlings, and a decrease in mean number of anal fin rays. Groups exposed to an intensity of 37 ft.-c. during age 8 to 13 days produced fingerlings with fewer vertebrae and dorsal fin rays than embryos reared in near-darkness of 0.02 ft.-c. or exposed to bright light. Embryos illuminated between blastopore-closure and pigmentation of the optic vesicles (age 15 to 20 days) were relatively unaffected at day 200 when compared to groups irradiated prior to closing of the blastopore; however, light-exposed embryos, when compared to controls, had reduced hatch, accelerated fingerling growth, and reduction in mean number of anal fin rays of surviving fingerlings. Embryos exposed to 37 ft.-c. during age 15 to 20 days produced fingerlings with fewer dorsal fin rays than embryos reared in near-darkness or exposed to bright illumination. Groups irradiated after formation of retinal pigments (days 22 to 27) were apparently not affected by hatching time; but, information is lacking on these groups after hatching. As was true in the previous experiment, pronounced differences were evident among progeny of different parents in practically every respect.

The processes by which visible radiation modifies various physiological systems of the salmon may be attributed to thermal, photochemical, and photoelectric properties of the experimental light source.

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BIOCHEMICAL GROWTH PATTERNS OF NORMAL AND RADIOTHYROIDECTOMIZED RATS*

J. ROSENTHAL AND F. DOLJANSKI

The Department of Experimental Medicine and Cancer Research, The Hebrew University —Hadassah Medical School, Jerusalem, Israel.

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The final size normally attained by an animal is the result of the rate and duration of growth, and is an inherent property of the organism. The cell population of each body part increases during development, reaching an adult level characteristic of the species. This level is possibly maintained by an equillibrium in which the number of cells produced equals the number lost. If, in the course of development, body growth becomes inhibited in one way or another, this could be the result of an inhibition either in the increase of cell number, or cell size, or both.

This paper represents an attempt to define which of the postulated factors could be responsible for growth inhibition caused by thyroid hormone deficiency.

It is a well-known fact that this deficiency results in an inhibition of body growth in mammals. If the thyroid gland is removed soon after it becomes functionally active, the full consequence of its absence becomes manifest. Such an experiment was for the first time performed by Salmon (24) on the new-born rat in which the thyroid becomes fully differentiated only 2-3 days before term (10). Salmon clearly demonstrated that body growth ceases completely at the age of about 3 weeks and the animals developed the typical symptoms of cretinism. In recent years it was established that in addition to the ablation of the gland, the dietary factor is of great importance in the experimental production of a full thyroid deficiency syndrome. The observation that thyroid hormone is present in food of animal origin (9) and the experiments by Purves and Griesbach showing that large doses of iodine prevent degranulation of the acidophil in thy-

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roidectomized rats (22) led Leblond and Eartly to suggest a diet eliminating all known sources of thyroxine and iodine to obtain the above-mentioned syndrome (15).

In the present experiments the thyroid gland of new-born rats was destroyed by radioactive iodine and the animals as well as the mother were maintained on an appropriate diet.

For the study of cell size and cell number, four organs representing three tissue types in the adult were chosen: the brain, as a typical postmitotic tissue, the liver and kidney showing only few mitoses, i.e. reverting post-mitotic and the stomach in which many mitoses are observed (3). The total organ DNA was used as an indicator of cell number and the protein/DNA ratio of cell size; RNA determination reflecting the protein synthetic capacity of the cells (16).

Materials and Methods

Albino rats from a local random-bred colony were radio-thyroidectomized according to the method of Goldberg and Chaikoff (8). Within 24 hours after birth the litters were weighed and injected with 100-130 μC of carrier-free I^{131} in 0.1 cc saline. The needle was introduced into the peritoneal cavity through the skin overlying the ribs, thus avoiding the loss of fluid, liable to occur in the usual mode of intraperitoneal injections. The normal control group was fed purina chow checkers (Ralston Purina Co., St. Louis, Mo.) and received tap water ad lib. Radiothyroidectomized rats (in the following referred to as "thyroidectomized") and the mother received a "Low Iodine" Test Diet (Nutritional Biochemical Co., Cleveland, Ohio) and distilled water ad lib. Control groups (thereafter referred to as "diet-controls") received the same diet plus 5 gamma iodine/1 cc, in the form of KI, in the drinking water. The animals were kept at a temperature of about 24°C.

All animals were weighed once a week, in the first three weeks the whole litter as a unit, and later on each animal individually. Of the 123 animals injected, 14 died, 56 were eaten up by their mothers and of the 53 left, only 17 were found to be completely thyroidectomized, the criterion for this being complete cessation of body growth. The thyroidectomized rats were very sensitive and required special care. The results are based on 47 normals, 19 diet controls and 17 completely thyroidectomized rats.

Chemical determination:

All animals were killed by decapitation and the organs weighed and frozen at —20°C. The stomach was rinsed in cold water before weighing. Prior to chemical determination, the organs were partially defrosted and homogenized in a Potter-Elvejhem homogenizer. All steps were carried out in the cold. For DNA, RNA & Protein determination a 10-20% homogenate was treated according to the Schneider procedure (25), the DNA was determined colorimetrically by the diphenylamine reaction, with the reagent freshly prepared for each determination. Color was allowed to develop for 18 hours at 37°C. RNA was determined by the Mejbaum method, as modified by Drury (4). Protein nitrogen was determined on the TCA precipitate by the Micro-Kjeldahl method of Koch and McMeekin (12). Dry weight was obtained by drying 1 ml. of the homogenate at 105°C. In all cases the values were within the normal range and are therefore not mentioned in the results.

All chemical determinations were carried out in duplicate.

Statistical analysis:

The values obtained for thyroidectomized animals were compared to those of normal animals of equal body weight by the t test of paired samples.

$$t_{n-1} = \frac{\bar{d}}{\sqrt{\frac{\Sigma d^2 - \bar{d}^2}{n(n-1)}}}$$

Nuclear size measurements were made on Zenker-fixed liver sections, stained with Haematoxylin-Eosin, and cut at 8 μ, 300 nuclei, randomly sampled, were drawn after microprojection, at a magnification of 1250. The longest and shortest nuclear diameters were measured and only those nuclei were used, where both diameters did not differ by more than 1 μ.

Results

Body growth:

Normal rats exhibit the typical sigmoid curve of growth. (Fig. 1 Normal Controls).

The Diet Controls showed retarded growth as compared with the

normal animals receiving a diet of purina chow checkers (Fig. 1 Diet Controls).

In the thyroidectomized animals body weight became stationary after 3-4 weeks (Fig. 1 Thyroidectomized).

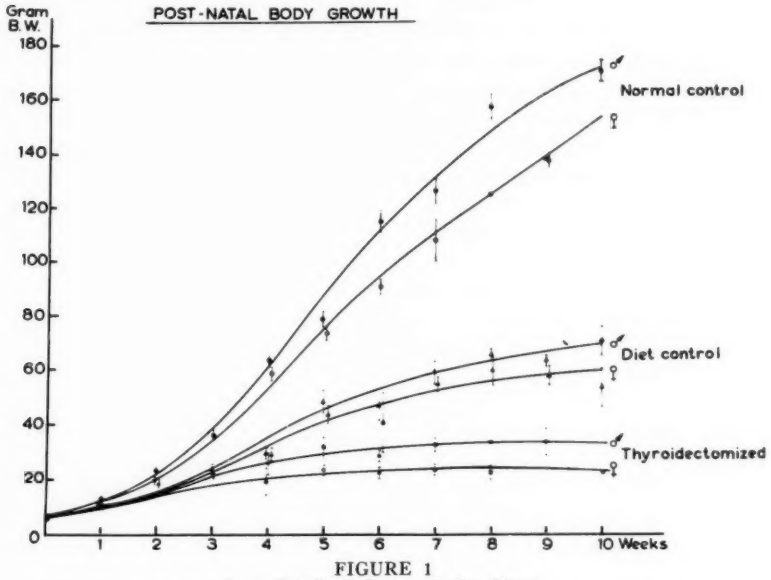


FIGURE 1

I = Standard Error of the Mean.

On the 4th week there is no significant difference between diet controlled and thyroidectomized animals. By the 5th week and further, the difference is highly significant ($p < 0.001$).

Organ growth:

Treatment of data: The values of organ weight, total DNA, RNA and protein content were plotted as a scatter diagram against body weight using a log-log scale. Within certain limits of body weight a linear relationship was found, being the expression of a simple allometric type of growth designated by the equation $y = ax^b$; where y is organ weight and x body weight. In the log-log plot this translates into $\log y = \log a + b \log x$, which gives a linear relationship between these 2 variables (23). The fitting of the regression line through the scattered points of the *normal* values was done by the

method of least squares (11). The algebraic expression of the slope is given by the value of *b*, i.e., the tangent of the angle which the line makes with the body weight axis. This value represents the ratio of the specific growth rates of the organ to that of the body as a whole.

Figures 2, 3, 4 and 5 show the relative growth of the liver, kidney, brain and stomach. It can be seen that each organ has its characteristic pattern of relative growth. However, the data show a common feature apparent in all four organs, namely: two phases of growth, a pre- and a post-weaning phase. Both phases are observed in respect to the organ growth as reflected in weight, as well as in the increase of DNA, RNA and protein content. The transitional period between the two phases, concomitant with the natural weaning of the animal, takes place when the animal attains a body weight of 15-25 gm. at the age of 2-3 weeks.

In the kidney, brain and stomach the relative growth decreases after weaning, i.e. during the second phase. (Kidney regression coefficient decreases from 1.2273 to 0.6540; Brain from 1.1733 to 0.0453; Stomach from 1.1009 to 0.7711). The only organ showing an increase in relative growth after weaning is the liver (from 0.8375 to 1.1782). In the latter the inflection point occurs earlier than in the other three organs, namely at the beginning of weaning (Fig. 2).

The differences in the specific growth rates of the constituents indicate the changes in the protein/DNA and RNA/DNA ratios of the organ. In the liver (Fig. 2) the protein/DNA ratio increases considerably both before and after weaning, reflecting the growth in size of the liver cell during post-natal development. RNA/DNA ratios increase during the first growth phase and remain almost constant thereafter.

In the kidney (Fig.3) weight, RNA and DNA have similar specific growth rates during the first phase. In the second phase weight and protein show a higher rate of increase as compared to that of DNA and RNA, thus indicating the gradual predominance of cell size as a factor in kidney growth during the second period.

In the brain, DNA shows during the first phase a relative growth rate lower than RNA and protein indicating a rapid increase in RNA and protein in the brain cell. In the second phase, growth practically ceases.

Only in the stomach specific growth rates of the four parameters

FIGURE 2

Regression lines of mg. liver weight, DNA-P, RNA-P and protein-N/liver (y) on gm. body weight (x) of normal rats.

dot : normal rat;
circle : thyroidectomized rats;
cross : diet controls.

regression equation ($\log y = b \log x + \log a$).

The regression line is drawn for the values of the normal rats only.

	Weight	DNA-P*	RNA-P	Protein-N
Phase I	B = 0.8375 Log A = 1.7092	B = 0.2685 Log A = 2.0861	B = 0.5057 Log A = 1.9625	B = 0.8303 Log A = 0.069
Phase II	B = 1.1782 Log A = 1.3150	B = 0.8688 Log A = 1.4918	B = 0.9480 Log A = 1.6581	B = 1.1754 Log A = 1.7577

Ratio of *b* of one component to *b* of the other component	RNA/DNA b b	Protein/DNA b b
Phase I	1.883	3.092
Phase II	1.091	1.352

* DNA Phosphorus.

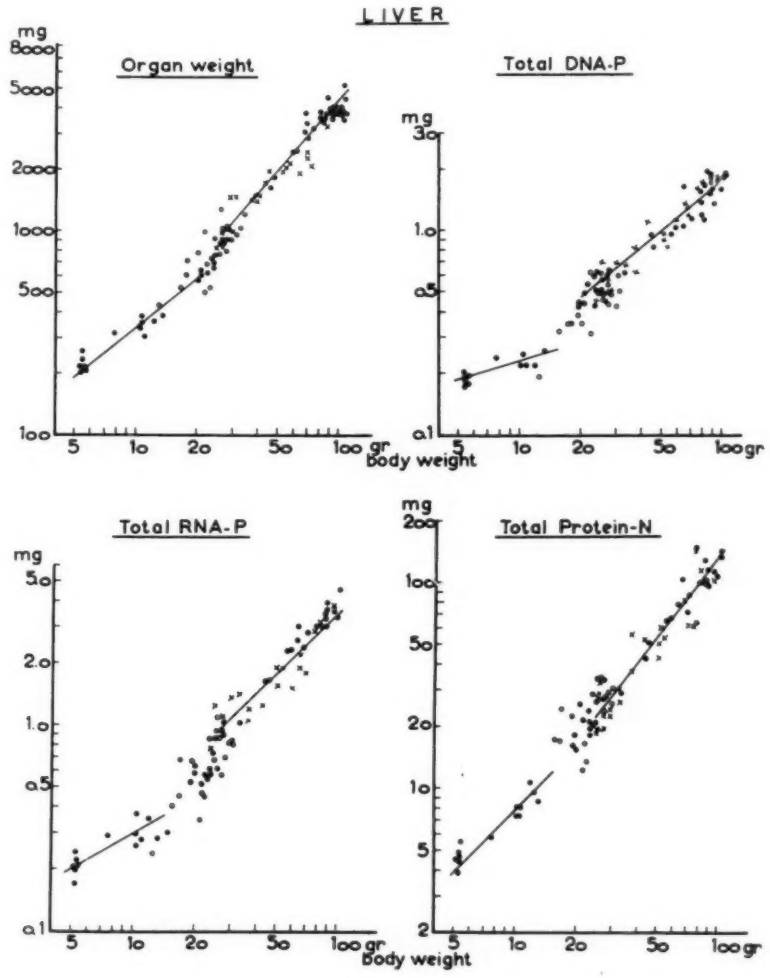

LIVER
Organ weight
mg
8000
5000
2000
1000
500
100
5
10
20
50
100 gr
Total DNA-P
mg
3.0
1.0
0.5
0.1
5
10
20
50
100 gr
Body weight
Total RNA-P
mg
5.0
2.0
1.0
0.5
0.1
5
10
20
50
100 gr
Total Protein-N
mg
200
100
50
20
10
5
2
5
10
20
50
100 gr
Body weight

FIGURE 3

Regression lines of mg kidney weight, DNA-P, RNA-P and protein-N/kidney (y) on gm. body weight (x) of normal rats.

dot : normal rat;
circle : thyroidectomized rats;
cross : diet controls.

regression equation ($\log y = b \log x + \log a$).

The regression line is drawn for the values of the normal rats only.

	Weight	DNA-P*	RNA-P	Protein-N
Phase I	B = 1.2273 Log A = 0.7974	B = 1.2016 Log A = 0.8234	B = 1.1037 Log A = 0.6296	B = 1.5517 Log A = 1.5968
Phase II	B = 0.6540 Log A = 1.6192	B = 0.2455 Log A = 2.2218	B = 0.2291 Log A = 2.0386	B = 0.7641 Log A = 1.7701

Ratio of *b* of one component to *b* of the other component	RNA/DNA b	Protein/DNA b
Phase I	0.918	1.291
Phase II	0.933	3.112

* DNA Phosphorus.

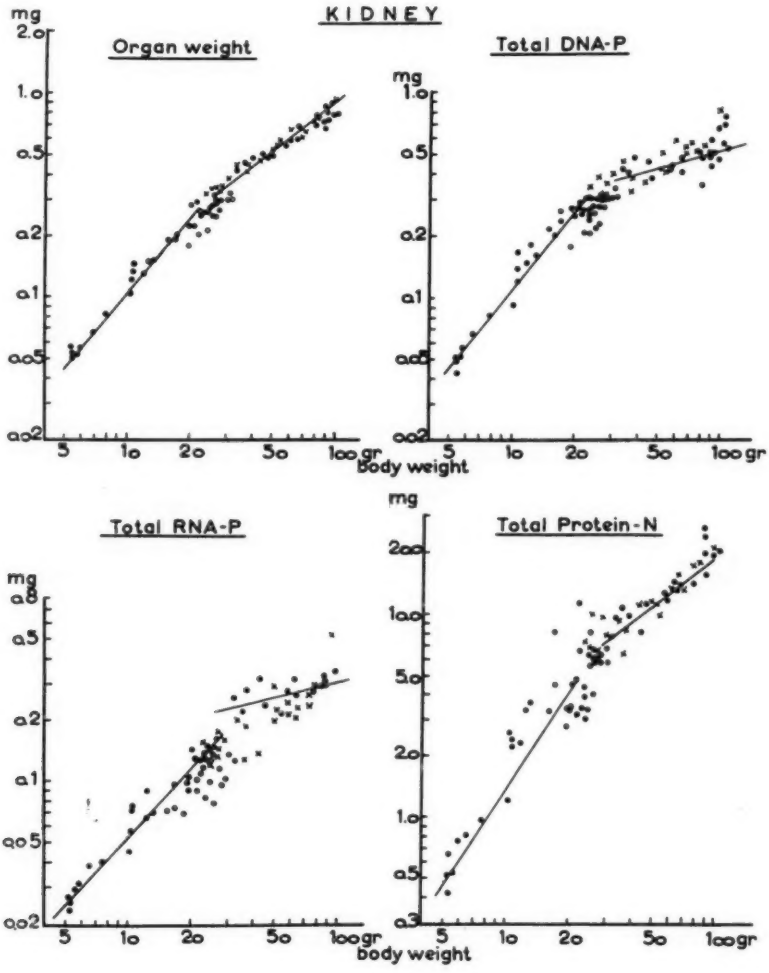

KIDNEY
Organ weight
mg
2.0
1.0
0.5
0.2
0.1
0.05
0.02
5
10
20
50
100 gr
Total DNA-P
mg
1.0
0.5
0.2
0.1
0.05
0.02
5
10
20
50
100 gr
body weight
Total RNA-P
mg
0.8
0.5
0.2
0.1
0.05
0.02
5
10
20
50
100 gr
Total Protein-N
mg
200
100
50
20
10
5
3
5
10
20
50
100 gr
body weight

FIGURE 4

Regression lines of mg brain weight, DNA-P, RNA-P and protein-N/brain (y) on gm. body weight (x) of normal rats.

dot : normal rat;
circle : thyroidectomized rats;
cross : diet controls.

regression equation ($\log y = b \log x + \log a$).

The regression line is drawn for the values of the normal rats only.

	Weight	DNA-P*	RNA-P	Protein-N
Phase I	B = 1.1733 Log A = 1.4858	B = 0.6480 Log A = 1.4234	B = 1.2655 Log A = 0.7487	B = 1.1808 Log A = 2.2849
Phase II	B = 0.0453 Log A = 3.0346	B = 0.2369 Log A = 2.0612	B = 0.2137 Log A = 2.0186	B = 0.1647 Log A = 0.8334

Ratio of *b* of one component to *b* of the other component	RNA/DNA b	Protein/DNA b
Phase I	1.952	1.822
Phase II	0.902	0.695

* DNA Phosphorus.

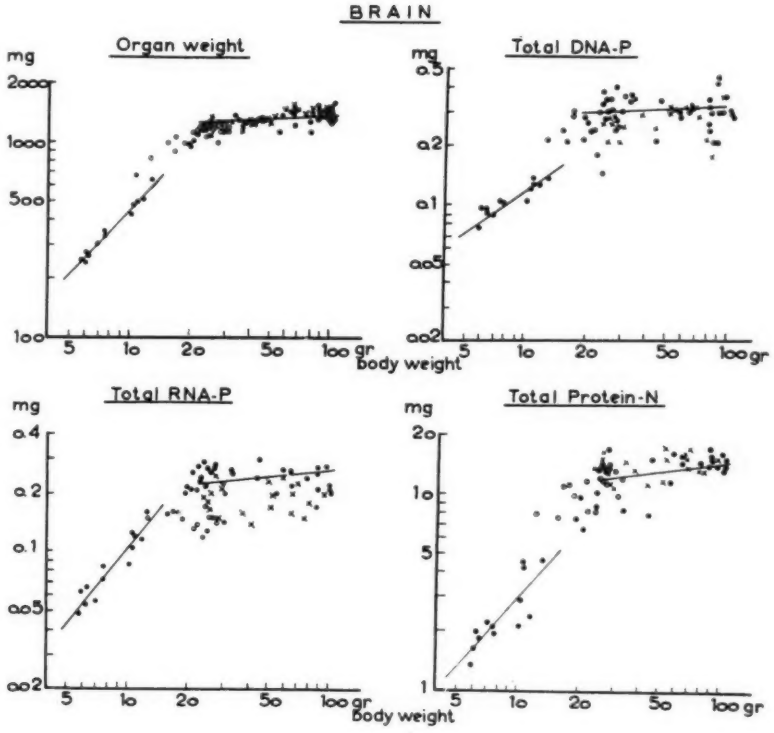

BRAIN
Organ weight
mg
2000
1000
500
100
5
10
20
50
100 gr
Total DNA-P
mg
0.5
0.2
0.1
0.05
0.02
Body weight
Total RNA-P
mg
0.4
0.2
0.1
0.05
0.02
Total Protein-N
mg
20
10
5
1
Body weight

FIGURE 5

Regression lines of mg. stomach weight, DNA-P, RNA-P and protein-N/stomach (y) on gm. body weight (x) of normal rats.

dot : normal rats;
circle : thyroidectomized rats;
cross : diet controls.

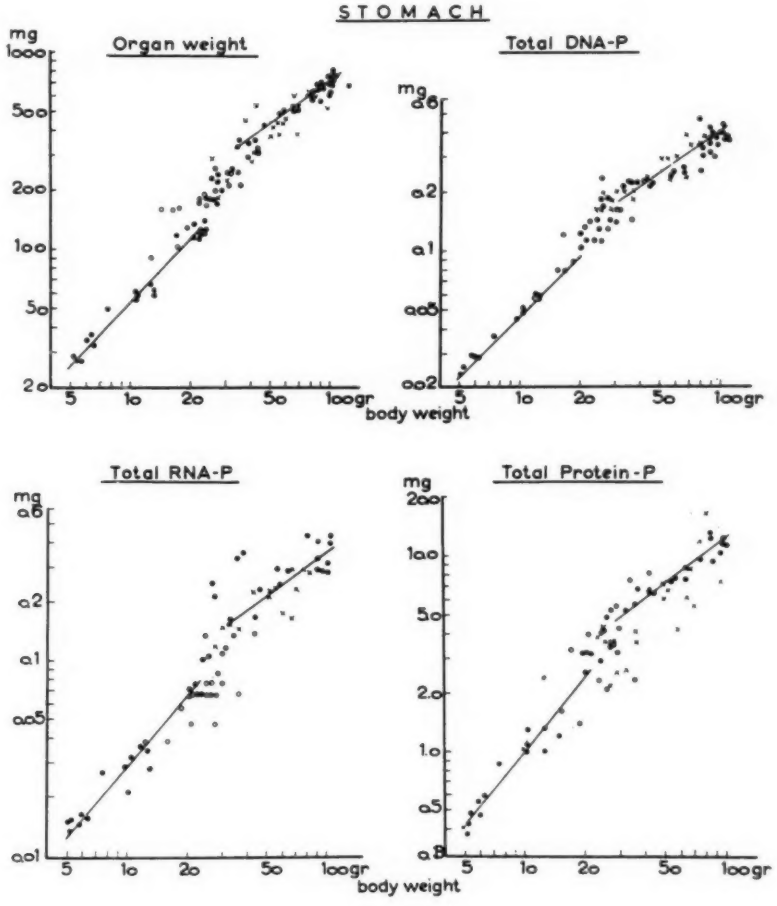
regression equation ($\log y = b \log x + \log a$).

The regression line is drawn for the values of the normal rats only.

	Weight	DNA-P*	RNA-P	Protein-N
Phase I	B = 1.1009 Log A = 0.6514	B = 1.1212 Log A = 0.5642	B = 1.1986 Log A = 0.2719	B = 1.3202 Log A = 0.7199
Phase II	B = 0.7711 Log A = 1.2965	B = 0.6912 Log A = 1.2288	B = 0.7164 Log A = 1.1312	B = 0.7487 Log A = 1.5876

Ratio of *b* of one component to *b* of the other component	RNA/DNA b b	Protein/DNA b b
Phase I	1.069	1.177
Phase II	1.036	1.083

* DNA Phosphorus.


STOMACH
Organ weight
mg
1000
500
200
100
50
20
5
10
20
50
100gr
body weight
Total DNA-P
mg
0.5
0.2
0.1
0.05
0.02
Total RNA-P
mg
0.5
0.2
0.1
0.05
0.01
Total Protein-P
mg
200
100
50
20
10
5
2

examined ran parallel courses before and after weaning, i.e., the protein/DNA and RNA/DNA ratio did not change. During the transitional period a short phase of accelerated growth took place (Fig. 5).

At ages varying from 4-10 weeks the thyroidectomized animals as well as the diet controls show organ weight, DNA and protein values as expected from their body weight. Conversely, the RNA values are decreased in the brain and kidney (level of significance $p = 0.05$), but not in the liver. A similar trend, but to a lesser degree, could be observed in the diet controls. In general the biological variation in thyroidectomized animals was wider than in the 2 control groups.

In a study of the nuclear size distribution in the liver, one nuclear class only, of the diploid type, could be found in the thyroidectomized rats at the age of 10 weeks. This nuclear size distribution corresponds to that observed in the two weeks old normal rats. It does, however, not tally with the distribution in the 10 weeks age group, where a new class of the tetraploid type has already appeared, which comprises about 70% of the liver cells. In other words, while the nuclear size distribution correlates with body weight it does not so correlate with chronological age.

Discussion

The growth of the body:

In thyroidectomized rats complete cessation of body growth occurs at the age of 3-4 weeks, in accordance with the findings of Salmon (24) and of Goldberg & Chaikoff (8). The same results are obtained in the young adult rat 3-4 weeks after the operation (15). This phenomenon is assumed to be caused by an inhibition of the synthesis and/or of the release of growth hormone from its putative source, the acidophils (5). The histological changes occuring in the pituitary of the thyroidectomized animal, as described by Purves and Griesbach (22), provided the morphological basis for the above assumption. These authors showed that whereas pituitary basophils respond in number and size even to slight lowering in the level of thyroid hormone production, total degranulation of the acidophils occurs only in response to *complete* thyroid deficiency. However, a dose of 0.25 micrograms of thyroxin was enough to prevent this degranulation, more so, as recently demonstrated by Evans, Rosenberg & Simpson

(6), such a dose restored body growth to almost normal. The above results led to the realization that cessation of body growth is a most reliable criterion for complete thyroid deficiency. If a slight increment in the body weight of such animals does occur, as described by Scow, Simpson, Asling, Li & Evans (26) this would indicate that some thyroid hormone of either endogenic or exogenic source was still in existence, an assumption corroborated by the presence of some acidophils in the pituitary of thyroidectomized animals (13).

The retardation in growth observed in the diet controls suggests a diet deficiency probably due to the low dietary value of gluten as the protein source.

The growth of organs:

The constant ratio prevailing—within certain body weight limits—between the specific growth rates of body parts and the body as a whole, is a phenomenon widely described and amply theorized upon (23).

The results of the present investigation indicate a similar behavior on the part of the major chemical constituents of the organs. The observed regression lines illustrate two growth phases with a transition during the weaning period, the latter occurring when the animals are 3 weeks old and have a body weight of 20-30 gms. The occurrence in the rat of a pre- and post-weaning growth phase was demonstrated and discussed by Zucker, Hall, Young and Zucker (28) and the importance of the weaning period as a critical stage in development was stressed by McDowell et al. (18). Bertalanffy & Pirozynski (1) who studied the relative growth of liver, brain and kidney in respect to weight increment found only one phase of growth up to a body weight of 100 gms. The observation of the pre-weaning period seems to have been omitted by these authors, probably due to the paucity of data recorded for that phase.

The growth pattern of the four organs studied shows that each has its own type of allometric growth.

The liver was the only organ in which an increase of relative growth occurred at weaning. Campbell and Kosterlitz (3) showed a constant growth ratio between liver constituents and body weight, but in a range of body weights differing from those reported here. The difference in the slope of the regression lines of the constituents reflects an

increase in cellular RNA and protein content, which is in accordance with the results of Fukuda and Sibatani (7).

In the kidney, relative growth decreased at weaning. The rise in DNA occurring after weaning indicates that, at least up to 100 gms., body weight, cell multiplication contributes to organ growth. This tallies with the findings of Kurnick (14). Furthermore, the differences in the growth ratio of protein to DNA, in the course of both growth phases, point to a continuous increase in cell size as the predominant factor responsible for organ growth. Evidently, nucleocytoplasmic relationships vary in the course of development.

The brain exhibited almost complete cessation of growth of all constituents during the second phase. It is well known that in the brain cell number reaches its final adult level soon after birth (19). The still apparent minimal rise in constituents can be accounted for by the growth of the mantel layer of the cerebrum and neuroglia (21).

In the thyroidectomized animals, weight, protein and DNA values of the examined organs were as predicted from body weight. The deviating constituent, exhibiting lower values, was the RNA of brain and kidney. This deviation could partly be accounted for by the diet, since a similar trend was observed in the diet controls.

Data obtained for thyroidectomized animals show that at the age of 5, 7 and 10 weeks organ weight, cell number and size reach values characteristic for a 3 weeks old normal animal of similar body weight. Thus, from the point of view of organ to body relationship, the body weight could be looked upon as a reflection of "physiological age" and consequently the thyroidectomized animal could be considered as reaching the physiological age of only 3 weeks in a lifetime of 7-10 weeks.

Maintenance of the proportions by the thyroidectomized and by the diet-control animals reveals that neither partial retardation or complete cessation of growth can exert an influence on the relation between organ and body,—at least in respect of the organs studied. This immutability of the relative growth values was also observed under conditions of controlled selection applied in the production of strains of small and large mice (17).

It would thus appear that the mechanism controlling the maintenance of proportions is of a very stable genetic character, unaffected by diet or hormonal variations.

Considering the pattern of growth brought to light in the present work, the phenomenon of cell constancy, as described by Eric Martini (20) at the beginning of this century is called to mind. The concept of cell constancy was derived from the phenomenon observed in several metazoan groups, where a constant number of cells, characteristic for the species, was found in each body part. Demonstration of this phenomenon was possible owing to the small size of the organisms (e.g. the rotatoria have only 1,000 cells). It was later suggested that the phenomenon of cell constancy might be more widespread, occurring in body parts of a large number of additional species. Also it was assumed to exist in the mammalian brain and heart (27). The maintenance of a level of cell population, typical for each organ, and characteristic for the species, suggests the possibility of broadening the concept under discussion so that it would comprise most of the species and thus acquire a much wider biological significance.

The constancy of proportion observed at various stages of development points to the concept of cell constancy in its wider application as being characteristic not only of the adult phase, but of each phase in growth.

Summary

1. A study of the growth of four organs, (liver, kidney, brain and stomach) in respect to their weight, DNA, RNA and protein content, was made in normal and in radiothyroidectomized rats.

2. It was found that within certain body weight limits, a constant ratio prevails between organ and body weight, as well as between total organ DNA, RNA and protein content and body weight.

3. Each organ showed a characteristic pattern of growth in cell number and cell size.

4. All organs showed an inflection point in the relative growth, during the third week of life.

5. On the whole, thyroidectomized animals showed values expected from their body weight except for RNA in the brain and kidney which was decreased. Hence these animals reach at an age of 10 weeks the development of a 3 week old normal rat.

6. The significance of these results is discussed.

Acknowledgement

We wish to thank Professor Jack Gross for his criticism and helpful discussions.

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GROWTH OF ASCITES TUMORS IN MICE INJECTED WITH X-IRRADIATED CELLS BY DIFFERENT ROUTES AND IN DIFFERENT DOSES[1]

JOHN R. MITCHELL, M.D.,[2] AND DAVID M. DONALDSON, PH.D.[3]

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INTRODUCTION

In a previous study (Donaldson and Mitchell, 1959) it was found that lethally x-irradiated Ehrlich's ascitic carcinoma when injected intraperitoneally had a definite influence on the growth of viable Ehrlich's ascitic tumor cells in the mouse. This paper reports further effects on the growth of Ehrlich's tumor when lethally x-irradiated ascitic cells are injected by different routes and in different doses.

METHODS AND MATERIALS

The ascites tumors were propagated in Swiss mice by intraperitoneal transfer of 0.1 ml. ascites fluid diluted in 1 ml. physiological saline. Tumor cells used were prepared as follows: 5 units of heparin/ml. were added to 20 ml. of asictes fluid from two or more donor mice. The heparinized ascites fluid was exposed to a dose of 10,000 r. x-irradiation in a sterile plastic petri dish and diluted with physiological saline solution to give the desired cellular concentration. The materials and methods used for counting the ascitic cells were the same as used in white cell counts. The X-ray factors were 250 K.V., 15 Ma, hvl 0.8 mm. Cu, tsd 40 cm., output 280 r/min. In all cases the tumor cells employed were collected and injected into recipient mice within one hour from the time of collection. The location of the transplant and number of cells used varied in the different experiments.

In a previous study (Donaldson and Mitchell, 1959), it was found that when lethally x-irradiated Ehrlich's ascitic carcinoma cells were injected intraperitoneally one day after a subcutaneous transplant of

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[2] Department of Radiology, Yale University Medical School, New Haven, Connecticut and the Radiologic Service of The Veterans Administration Hospital, West Haven, Connecticut.

[3] Department of Bacteriology, Brigham Young University, Provo, Utah.

16,500 cells from an Ehrlich's ascites carcinoma, the mortality decreased from 70% to 7%. The experiments reported here were designed to study the effects upon growth of Ehrlich's ascites tumor when x-irradiated cells from the same tumor when injected in different routes and in different doses.

Results

Rejection of Subcutaneous Tumor

Starting one day after the subcutaneous transplant of 200,000 viable untreated tumor cells/mouse, each mouse in the experimental group received an intraperitoneal injection of 200,000 tumor cells that had been exposed to 10,000 r x-irradiation *in vitro*. This injection was followed by five similar injections of x-irradiated cells at two day intervals. The control group of mice received the challenge transplant of 200,000 cells, but no x-irradiated cells. The mortalities for this experiment and the previously reported experiment are compared in text-figure 1. Although the degree of growth inhibition induced by x-irradiated tumor cells was not as great following the massive challenge the decrease in mortality following this tumor transplant was statistically significant as is shown in the first part of Table I.

Dramatic differences in gross appearance of the tumors in the different groups were observed. In contrast to the lack of tumor growth when the immunization followed subcutaneous grafts of 16,500 cells, the animals receiving 200,000 viable untreated cells did develop tumors. The appearance of tumors is similar to the tumors growing in control mice until approximately one week after the last injection of x-irradiated cells. At this time the tumors of the experimental mice became inflamed and ulcerated. Liquefaction occurred at the periphery of the tumor, and the solid central mass of the tumor was rejected.

Enhancement of Intraperitoneal Tumor Growth

When x-irradiated cells were injected intraperitoneally after the inoculation of viable cells by the same route, there was no protection. Instead a stimulating effect on the growth of the tumor and more rapid death of the host was noted and is recorded in text-figure 2.

Passive Immunization with Serum

Since some protection was induced by active immunization against

TABLE I

SUMMARY OF THE ACTIVE AND PASSIVE IMMUNIZATION EXPERIMENTS AGAINST EHRLICH'S ASCITES CARCINOMA

Experiment	Group	Immunization	Mortality		P value*
			Ratio	Per cent	
1. Rejection of sc transplants following active immunization	Control	None	42/43	98	< 0.001
	Immune	5 ip injections of x-irradiated* cells starting one day after challenge transplant	34/55	62	
2. Passive immunization with serum	Control	None (ip injection of normal serum)	29/29	100	< 0.05
	Immune (serum)	ip injection of 0.5 ml sera from immunized mice ½ hour before challenge transplant	13/17	76	
3. Passive immunization with cells	Control	None	22/22	100	> 0.9
	Immune (cells)	Cells from immune animals injected ½ hour before challenge transplant	24/24	100	
4. Immunization by iv injection of x-rayed or normal tumor cells	Control	None	29/29	100	< 0.001
	Immune I (x-rayed tumor cells)	iv injection of 2,000,000 x-irradiated cells† 15 days before challenge transplant	16/40	41	
	Immune II (normal tumor cells)	iv injection of 2,000,000 normal tumor cells 15 days before challenge‡	12/35	34	< 0.001

* P values calculated by comparing immune groups with their respective controls using Chi Square.
† Cells used for immunization received a 10,000 r x-ray dose *in vitro*.
‡ Tumors do not develop in normal mice when viable tumor cells are injected by the iv route.

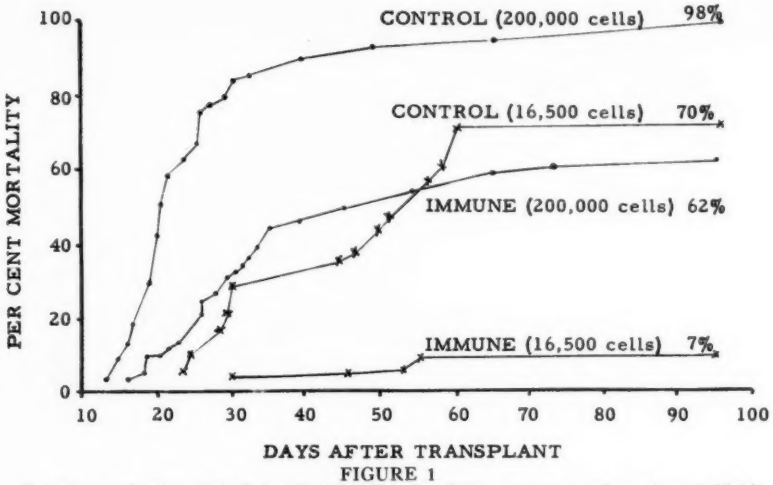

FIGURE 1

Post-Immunization with X-irradiated Cells after Subcutaneous Implantation of Viable Ehrlich's Ascites Tumor Cells. Numbers of Tumor Cells Transplanted are in Parenthesis.

INTRAPERITONEAL X-IRRADIATED CELL AFTER VIABLE CELLS

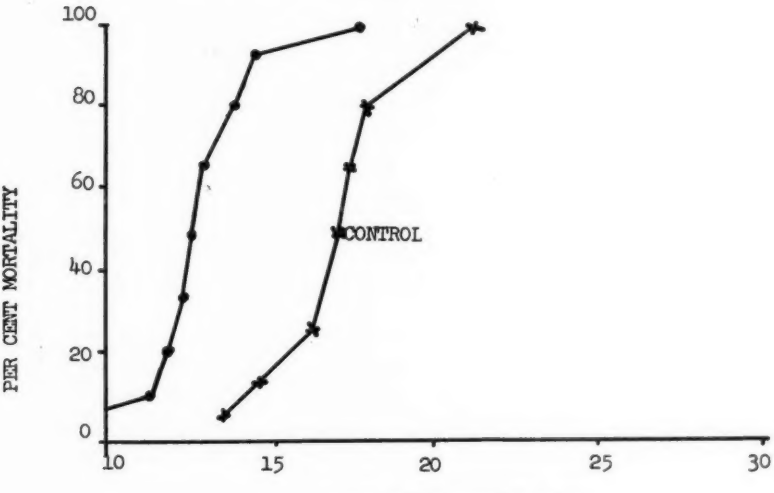

FIGURE 2

Post-Immunization with X-irradiated Cells after Intra-peritoneal Injection of Viable Ehrlich's Ascites Tumor Cells.

subcutaneous Ehrlich's Ascites tumor grafts, the possibility of obtaining passive immunity was considered.

Serum pools were collected from non-immunized mice and mice that had been immunized and survived a challenge with viable untreated tumor cells. Cardiac punctures were made on the immunized mice 39 days after the tumor graft had been attempted. One half hour after an 0.5 ml. intraperitoneal injection of serum from either immunized or normal mice, 20,000 viable untreated tumor cells were placed in the peritoneal cavity of each mouse. The daily mortalities of these mice are recorded in text-figure 3. The mortality of the serum treated mice was reduced to 76%. All control mice died.

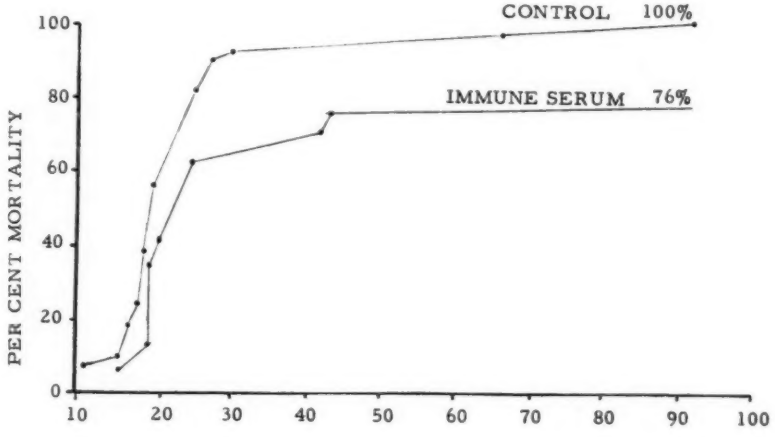

FIGURE 3
Passive Immunization with Sera from Immunized Mice.

Passive Immunization with Cells

In order to investigate whether or not cellular immunity against this tumor was of major importance, cells were collected from the peritoneal cavity of animals that had been immunized and had survived a challenge with viable tumor cells. This was done by sacrificing the immune animals and washing the peritoneal cavity with 5 ml. of saline. Microscopic examination of the peritoneal washings demon-

strated endothelial cells, erythrocytes, lymphocytes, leukocytes and atypical cells that were thought to be ascitic tumor cells. Two and one-half ml. of the peritoneal washings were then injected into the peritoneal cavities of normal non-immunized mice. One to four hours later these cell-treated animals received an intraperitoneal injection of 20,000 live tumor cells. The mice which served as controls received the live tumor cells and the peritoneal washings from the normal animals. The results of this experiment are summarized in the third part of Table I. No differences were observed in the survival times, and the final mortality was 100% in both groups.

Effects of Normal and X-irradiated Tumor Cells Via the Intravenous Route

A group of mice was treated with a single intravenous injection of 2,000,000 viable untreated tumor cells. A second group was treated with a single intravenous injection of 2,000,000 tumor cells that had been exposed to 10,000 r *in vitro*. A third group of mice was used as a control. Thirty days after the single injection all mice received an intraperitoneal challenge of 20,000 cells. As seen in text-figure 4, intravenous injections of either X-rayed or normal cells induced about

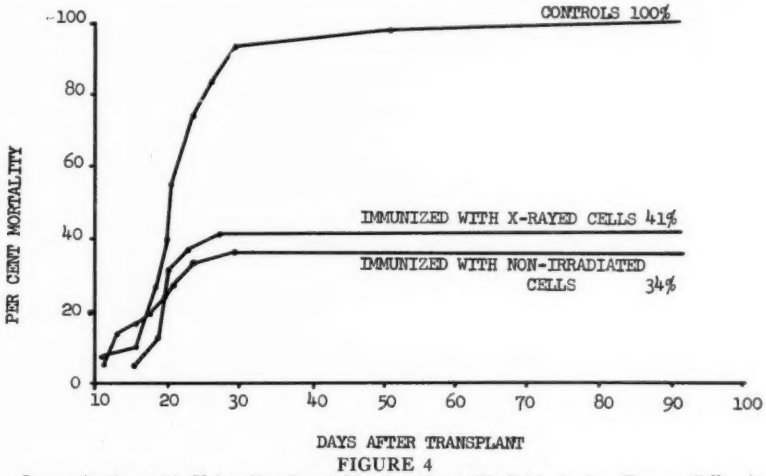

FIGURE 4

Immunization with X-irradiated and Non-irradiated Ehrlich's Ascites Tumor Cells via the Intravenous Route.

the same protection against a subsequent intraperitoneal injection of the untreated tumor cells.

Attempt to Reactivate Tumors in Immunized Mice that Survived Challenge Transplants

Tumors did not develop in normal mice injected with the peritoneal washings from animals that had survived an earlier intraperitoneal injection of viable Ehrlich's ascites tumor cells. Nevertheless, it was speculated that the tumors might reactivate if the host's resistance to the tumor decreased. To test this hypothesis, one group of mice received 5 micrograms of meticortelone daily for 30 days. This hormone treatment was started 119 days after the immunized animals had received a challenge injection of viable cells and showed no signs of tumor. The control groups of mice for these experiments consisted of immunized survivors that had received identical transplants. In no case did a tumor develop in either a control or hormone treated mouse. In the second such experiment mice received a 300 r dose of total-body x-irradiation 101 days after challenged with viable tumor cells. The X-ray factors employed were 250 KV, 15 ma, hvl 1.68 mm Cu, tsd 50 cm. Twenty-three days later these same animals received a second 300 r exposure. In no case did an irradiated mouse develop a tumor. The results in table II support the conclusion that the effects of immunization on the tumor transplant are lethal rather than static in nature.

Discussion

In both the intraperitoneal and the intravenous routes the recipient mice failed to react to the presence of tumor cells with the production

TABLE II
FAILURE OF HORMONE TREATMENT OR TOTAL-BODY X-IRRADIATION TO REACTIVATE TUMORS IN IMMUNIZED MICE

Group*	Treatment	Treatment started	Mortality	
			Ratio	Per cent
Control	None		0/20	0
Hormone	5 μg meticortelone/day for 30 days	119 days after transplant	0/18	0
Control	None		0/22	0
X-ray	Two 300 r total-body exposures 23 days apart	101 days after transplant	0/22	0

* Prior to use in these experiments all mice in control and treated groups were immunized and had survived a challenge with viable tumor cells.

of stroma and blood vessels. When cells were injected subcutaneously, solid tumors developed. When lethally x-irradiated cells were later injected intraperitoneally, the subcutaneous transplants were rejected, evidence that an immune reaction was involved. It was suspected that the immune reaction developed within the tumor bed itself. Further evidence that the tumor bed was the site of the immune reaction was noted in animals with grafts implanted into the brain and into the anterior chamber of the eye. These sites are isolated from the reticuloendothelial system, and rejection did not occur as in those animals that had subcutaneous implants.

Injection of x-irradiated cells intraperitoneally following the injection of viable cells into this space provoked an increase in tumor multiplication, growth, and more rapid death of the host. In a comprehensive review (Kaliss, 1958), has described a stimulating effect on tumor growth by injecting attenuated tumor cells, and others (Revesz, 1958), has shown that the proliferation of a small viable tumor graft may be stimulated by the presence of irreversible x-ray damaged tumor cells. It was speculated that the damaged cells were stimulating either by absorption of inhibitory agents, the addition of break-down products from the damaged cells necessary for growth of rapidly dividing cells, the maintenance of a normal ratio between dead and dividing cells, or a combination of these mechanisms.

When the intravenous route was used, no distinction was made between the lethally damaged x-irradiated cells and the viable tumor cells. It was postulated that the viable cells were soon damaged by the host and immunity responses from systemic effects occurred.

Summary

Susceptible animals, after receiving intraperitoneal inoculations of lethally x-irradiated Ehrlich's ascites carcinoma cells, became resistant to later intraperitoneal and subcutaneous injections of viable Ehrlich's ascites tumor cells. When x-irradiated cells were given after the subcutaneous implantation of viable Ehrlich's ascites tumor cells, rejection of the tumor occurred. X-irradiated cells, when injected after an intraperitoneal transplant, stimulated tumor growth rather than inhibiting it. A slight degree of passive protection was afforded by treatment with serum obtained from immunized mice. Transfer of peritoneal

cells from immunized to suceptible mice failed to protect from subsequent tumor grafts.

No differences could be demonstrated between viable tumor cells and x-irradiated tumor cells when injected intravenously. In a heterologous host tumor system such as the one used in this report, the intravenous route is the most heterologous. The subcutaneous route is intermediate in response and the intraperitoneal route is most isologous.

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Growth, 1961, **25**, 377-392

THE PROTEINS AND CARBOHYDRATES OF ASCITES TUMOR FLUID*

ELIZABETH ESHELMAN MILLER**

Bio-Research Institute, Cambridge 41, Mass.

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Since the discovery of the Ehrlich ascites carcinoma (6) and the demonstration by Klein and Klein (3, 4) that many solid tumors could be adapted to the ascites form of growth, ascites tumors have become important tools in cancer research. Work has been focused, however, on the ascites tumor cell, whereas the fluid surrounding the cell, the medium in which it grows, would be an ideal place to study substances produced by the tumor cell or utilized by it for growth.

In some respects, the ascites tumor fluid may be considered analagous to tuberculin, the liquid in which Mycobacterium tuberculosis is grown, whose proteins are of much diagnostic importance (13,14). The interplay of antibodies to the proteins and polysaccharides in tuberculin is significant in resistance to tuberculosis (20, 15, 18), and Seibert has based a theory of immunity in tuberculosis (17) on these facts. Some of the principles involved may also apply to other diseases, such as cancer. For example, substances produced by tumor cells, by interfering with the antibody mechanism, may be responsible for the lack of resistance of the tumor-bearing host to the growth of the tumor or its lowered antigenic response to other diseases or injected antigens (24).

It appears, therefore, to be of considerable interest to examine the nature of the proteins and carbohydrates in the fluid from ascites tumors. Since ascites fluid is known to be composed, at least in part, of material withdrawn from the peripheral circulation (21), a comparison must be made with serum from the ascites tumor-bearing host. This, in turn, must be compared with serum from normal animals or

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** Present address: Virus Research Laboratory, University of Pittsburgh School of Medicine, Pittsburgh 13, Pa.

those bearing the same tumor in the solid form, because the growth of the tumors themselves affects the serum compositon. Sarcoma I in A/Jax mice was chosen to initiate these studies, since plasma changes associated with the growth of this tumor have been defined (1, 10). The solid tumor was adapted to growth in the ascites form, and analyses for protein and carbohydrate were made on the ascites tumor fluid and various sera by electrophoretic and chemical methods. The results of these studies are described in the present paper.

Experimental

The adaption of Sarcoma I to grow in the ascites form was carried out according to the procedure described by Klein (3). After the first fluid transplant, the tumor has been carried by intraperitoneal inoculations of 0.4 cc of ascites fluid at six- to seven-day intervals. The remainder of the fluid from each transplant was freed from cells by two centrifugations at 1,500 rpm and frozen immediately to be used for chemical analysis. Serum from mice bearing the Sarcoma I ascites tumor, as well as from those with the same tumor in the solid form, was collected by severing the brachial plexus and withdrawing the blood with a pipette. The serum was also stored in the frozen state.

At various intervals the ascites tumor was tested for viability by subcutaneous inoculation of the ascites fluid into two mice. One of the resulting solid tumors was used for histological studies; the other was observed for the length of time it took to kill the mouse. Smears from the ascites tumor fluid itself were fixed by Carnoy's method and stained with hematoxylin and eosin.

Paper electrophoresis experiments were carried out in a horizontal strip apparatus n 0.1 μ, pH 8.6 veronal citrate buffer for six hours at a constant current of 16 ma. Ten and 20 lambda samples were applied with micropipettes to sheets of filter paper 12-3/4 x 7-1/4 in. divided by pencil lines into 1-1/2 in. strips. After completion of electrophoresis the filter paper sheets were dried at 60°-80°, and strips were cut apart for staining. Adjacent strips were stained with Ponceau 2 R by the method of Röttger (11) to demonstrate protein components, and with basic fuchsin by the method of Koiw and Grönwall (5) for protein-bound carbohydrates. For each of the samples analyzed, four replicate strips were stained by each method. The percentage of the

total represented by each component was determined by integration of tracings on a Spinco Analytrol densitometer. Values of the replicate determinations were then averaged, and these averages were used in the statistical calculation, which represents then the biological mean of differences between individual animals.

Chemical analyses for total protein and carbohydrate were carried out on each serum or ascites fluid used for electrophoresis. Determinations for protein were made by the Lowry method (7). Carbohydrate was determined with the use of the orcinol reagent in 70% H_2SO_4. Calculations were made for the amount of these substances in each electrophoretic component.

In some instances the ascites fluid was hemolyzed and the electrophoretic patterns were corrected in proportion to the amount of hemoglobin present, which was determined by reading diluted samples in a Klett colorimeter with No. 54 filter and comparing with a standard curve prepared from mouse hemoglobin. The influence of hemoglobin on the electrophoretic pattern was determined by paper electrophoretic analysis of samples of unhemolyzed ascites fluid before and after the addition of known amounts of hemoglobin to the samples.

Most experiments were carried out on ascites fluid or serum from individual animals. In addition, pools of the different fluids were made in order that quantitative dialyses could be carried out. Both electrophoretic experiments and chemical analyses were made on the pooled samples before and after dialysis. Average values based on these experiments were used for calculating carbohydrate content of electrophoretic components.

Results

Sarcoma I in A/Jax mice was found to be readily adaptable to growth in the ascites form in the abdominal cavity. At the six- to seven-day intervals of transplantation, three to five cc of fluid were regularly accumulated. The average survival time, calculated from 71 animals, was 12 days. Subcutaneous inoculation of fluid at various intervals resulted in solid tumors, histologically indistinguishable from the original Sarcoma I, which was maintained by trocar implantation. The ascites fluid itself contained free growing tumor cells and many mitotic figures, which may be seen in Figure 1, photographed from a smear taken from the 100th fluid transplant.[1]

[1] This smear was prepared by Mr. John Baker, Bio-Research Institute, Cambridge, Massachusetts.

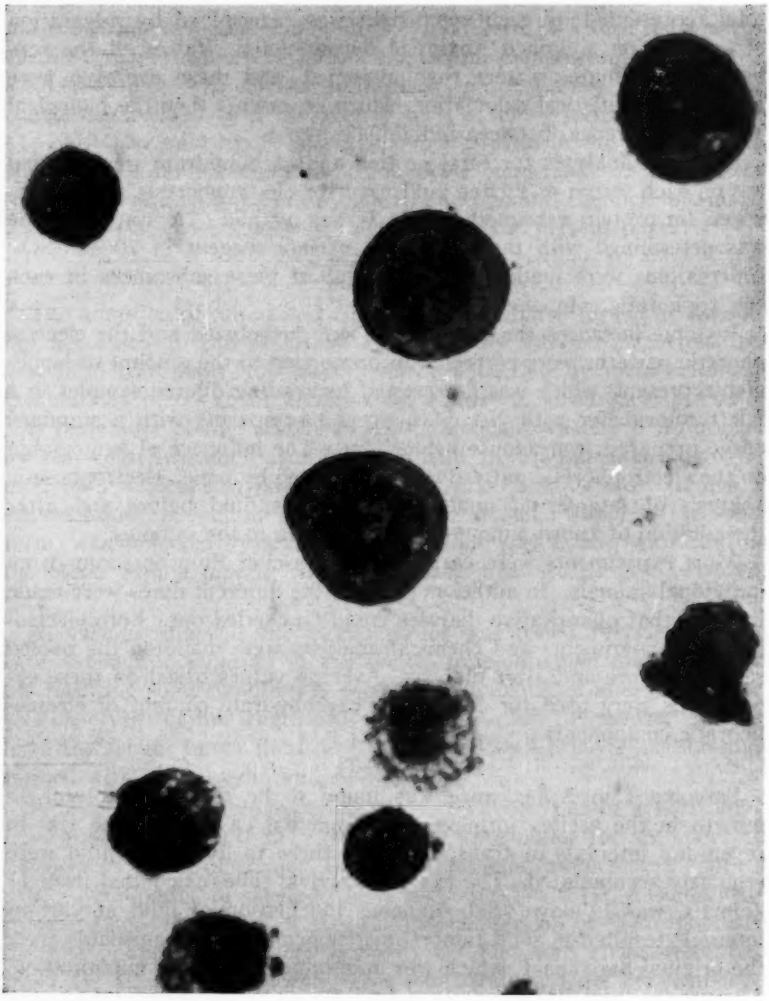

FIGURE 1

Sarcoma I ascites tumor cells. Smear made from 100th fluid transplant. Carnoy fixation, hematoxylin and eosin stain.

Paper electrophoresis analyses of the protein and carbohydrate components of Sarcoma I ascites tumor fluid showed that the composition of this fluid is different from serum of either normal or tumor-bearing mice. Line tracings of densitometer drawings of the electrophoretic patterns of SI asictes tumor fluid, serum from mice bearing SI in both the ascites and solid forms, and normal mouse serum are shown in Figure 2. These patterns show that, although in the ascites tumor fluid five components corresponding to those found in serum are resolved, the relative proportion of these components is different from that of any of the sera. The most outstanding difference is found in the α-globulin components. In the ascites tumor fluid the proportion of α_1- and α_2-globulins appear to be approximately equal in contrast to the serum α-globulins where α_2- greatly exceeds α_1-globulin.

The magnitude of the variations in proportion of these components may be seen more readily from calculations of the relative amounts of each component in percent of the total. Table I gives the relative percentages for each of the protein components along with the statistical significance of the differences between percentages of various components in the different fluids (t values). Considering a five percent level of significance, interesting comparative differences may be pointed out, which confirm the variations in patterns seen in Figure 2. Outstanding among these differences is the greater proportion of α_1-globulin and lower proportion of α_2-globulin found in the ascites tumor fluid than in the serum of the ascites tumor-bearing mice. In the serum of mice bearing both solid and ascites tumors, as well as in the ascites tumor fluid, the proportion of albumin is less and α_2- and β-globulin is greater than it is in normal serum.

The relative percents of the protein-bound carbohydrate components are shown in Table II. The differences found correspond in general to those found in the protein components, the α_1-globulin of the ascites tumor fluid showing a greater proportion of bound carbohydrate than serum from the ascites tumor-bearing mouse.

Analyses were carried out on ascites fluids taken over a period of time from the beginning of the adaptation of the tumor to the ascites form of growth through the 116th fluid transplant. During this time the total protein of the ascites fluid gradually increased from transplant to transplant. Although the total protein increased during this

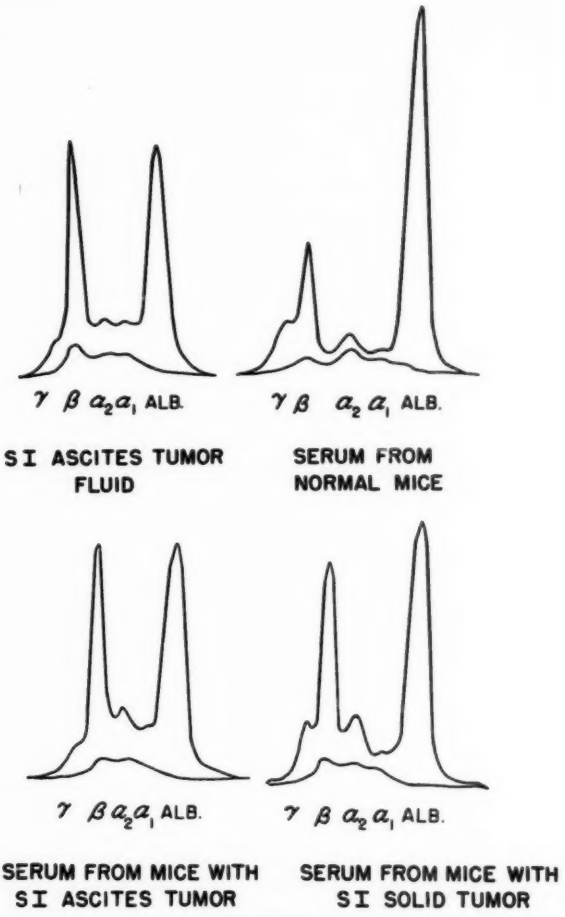
γ β a_2 a_1 ALB.
γ β a_2 a_1 ALB.
SI ASCITES TUMOR FLUID
SERUM FROM NORMAL MICE
γ β a_2 a_1 ALB.
γ β a_2 a_1 ALB.
SERUM FROM MICE WITH SI ASCITES TUMOR
SERUM FROM MICE WITH SI SOLID TUMOR

FIGURE 2

Paper electrophoretic patterns of Sarcoma I ascites tumor fluid, serum from normal A/Jax mice, serum from A/Jax mice bearing Sarcoma I ascites tumor or Sarcoma I solid tumor. Tracings of densiometric drawings of paper strips; upper curves show protein components taken from strips stained with Ponceau 2 R. Lower curves show carbohydrate components taken from strips stained by the Feulgen technique.

TABLE I

COMPARISON OF RELATIVE PERCENTS OF PROTEIN COMPONENTS OF SI ASCITES TUMOR FLUID, SERUM FROM MICE BEARING SI ASCITES AND SI SOLID TUMORS AND NORMAL MOUSE SERA

a. Electrophoresis Data

Material analyzed	No. of samples**	Percent of total area*				
		Albumin	α_1	α_2	β	γ
SI ascites tumor fluid	16	43.5 (2.9)	11.3 (2.8)	11.6 (1.5)	24.8 (2.5)	8.8 (1.3)
Serum from mice with SI ascites tumor	7	43.6 (4.1)	7.3 (1.2)	15.0 (2.0)	26.4 (3.1)	7.7 (1.0)
Serum from mice with SI solid tumor	6	48.9 (4.1)	5.7 (1.0)	11.5 (1.0)	23.3 (1.5)	9.7 (1.8)
Serum from normal A/Jax mice	6	59.5 (4.2)	5.6 (1.3)	10.0 (1.1)	16.5 (1.9)	8.4 (1.5)

b. T Values

Fluids compared	T value				
	Albumin	α_1	α_2	β	γ
SI ascites tumor fluid with serum from mice with SI ascites tumor	—0.03	+3.6	—4.5	—1.3	+2.0
SI ascites tumor fluid with normal serum	—10.2	+4.8	+2.3	+7.2	+0.6
Serum from mice with SI ascites tumor with normal serum	—6.9	+2.5	+5.5	+6.8	—1.0
Serum from mice with SI solid tumor with normal serum	—4.4	+0.2	+2.5	+7.0	+1.4
Serum from mice with SI ascites tumor with serum from mice with SI solid tumor	—2.3	+0.7	+3.9	+2.3	—2.0

* Figures in parentheses represent standard deviation of the mean.

** Each sample represents the average of 3 to 4 determinations on an individual serum.

TABLE II

COMPARISON OF THE RELATIVE PERCENTS OF CARBOHYDRATE COMPONENTS OF SI ASCITES TUMOR FLUID, SERUM FROM MICE BEARING SI ASCITES TUMORS AND SI SOLID TUMORS AND NORMAL MOUSE SERUM

a. Electrophoresis Data

Material analyzed	No. of samples**	Percent of total area*				
		Albumin	α_1	α_2	β	γ
SI ascites tumor fluid	17	18.2 (4.6)	22.9 (4.4)	24.7 (4.0)	28.8 (7.0)	8.4 (3.1)
Serum from mice with SI ascites tumor	7	17.5 (3.6)	14.0 (2.9)	30.9 (4.7)	29.3 (4.7)	8.4 (2.7)
Serum from mice with SI solid tumor	6	15.5 (3.3)	12.7 (2.8)	32.8 (4.1)	31.6 (4.3)	7.3 (1.7)
Normal mouse serum	5	22.9 (3.3)	14.8 (4.5)	33.7 (4.0)	18.7 (1.8)	10.0 (11.0)

b. T Values

Fluids compared	T value				
	Albumin	α_1	α_2	β	γ
SI ascites tumor fluid with serum from mice with SI ascites tumor	+0.46	+4.9	−3.3	−0.2	±0
SI ascites tumor fluid with normal serum	−2.1	+3.6	−4.4	+3.2	−0.5
Serum from mice with SI ascites tumor with normal serum	−2.7	−0.4	−1.1	+4.8	−0.4
Serum from mice with SI solid tumor with normal serum	−3.2	−0.9	−0.4	+6.1	−0.6
Serum from mice with SI ascites tumor with serum from mice with SI solid tumor	+1.0	+0.8	−0.8	−0.5	+0.9

* Figures in parentheses represent standard deviation of the mean.

** Each sample represents the average of 3 to 4 determinations on an individual serum.

time, the relative proportion of the components present remained constant, and the electrophoretic pattern characteristic of the ascites fluid was evident even in the early transplants.

These differences in relative proportion of components cannot be interpreted on an absolute basis, however, but must be considered with respect to the amount of protein and carbohydrate present. The actual amount of protein and carbohydrate in each component, as well as the total, is presented in Tables III and IV. It may be seen from these Tables that both total protein and carbohydrate are lower in the ascites tumor fluid and sera from tumor-bearing mice than in the normal serum.

Table III shows that the low total protein in the ascites fluid and tumor sera is due to a large extent to a reduction in the amount of albumin. The β-globulin, on the other hand, appears to be increased. This table also shows that there is more α_1-and less α_2-globulin in the ascites fluid than in circulating serum from mice bearing the ascites tumor. These differences can be seen more clearly if the values for the ascites tumor fluid are multiplied by a factor which brings them to the protein level of the circulating serum, as seen in line 2 of Table III and Table IV.

Table IV shows that ascites tumor fluid has more carbohydrate bound to the α_1- and β-globulins than is found in the serum from the ascites tumor-bearing mice. The lower total carbohydrate of both types of tumor sera, as well as the ascites tumor fluid, was reflected in all of the components except the β-globulin.

The above experiments were carried out on samples of serum and ascites fluid collected from individual mice. However, in addition, pools of the various sera and ascites fluids were also used, so that large enough samples could be obtained to determine quantitatively how much carbohydrate was lost during dialysis. It soon became evident that the total protein was also lower after dialysis. Table V shows total protein of the ascites fluid and various sera before and after dialysis for 24 hours against 0.85% NaCl. The amount of protein, or material producing color with Lowry reagent, which dialyzed averaged about 10% of the total, and there was no consistent variation among the ascites tumor fluid or sera. Recovery and analysis of the dialysate showed that the loss was due to material which gave color with the Lowry reagent, but which did not take the protein stain after applica-

TABLE III

COMPARISON OF AMOUNT OF PROTEIN IN SI ASCITES TUMOR FLUID, SERUM FROM MICE BEARING SI ASCITES TUMORS AND SI SOLID TUMORS, AND NORMAL MOUSE SERUM

a. Electrophoresis Data

Material analyzed	No. of samples	Mg per cc*					
		Total protein	Albumin	α_1	α_2	β	γ
SI ascites tumor fluid	17	54.9 (10.6)	24.1 (5.4)	6.0 (1.5)	6.4 (1.4)	13.5 (3.0)	4.9 (1.4)
Ditto (corrected)**		64.4	28.2	7.0	7.5	15.8	5.7
Serum from SI ascites tumor	7	64.4 (8.0)	28.0 (3.5)	4.7 (1.2)	9.8 (2.2)	17.0 (3.0)	4.9 (2.2)
Serum from SI solid tumor	6	75.5 (13.4)	37.4 (5.3)	4.4 (1.3)	8.7 (1.7)	17.7 (4.0)	7.5 (2.4)
Normal serum	6	86.8 (4.6)	51.5 (2.7)	4.9 (1.3)	8.7 (1.2)	14.3 (2.1)	7.3 (1.5)

b. T Values

Fluids compared	T value				
	Albumin	α_1	α_2	β	γ
SI ascites tumor fluid with serum from mice with SI ascites tumor	—1.7	+2.0	—4.6	—2.6	±0
SI ascites tumor fluid with normal serum	—11.7	+1.6	—3.8	—0.6	—3.5
Serum from mice with SI ascites tumor with normal serum	—13.4	—0.2	+1.0	+1.8	—3.7
Serum from mice with SI solid tumor with normal serum	—5.8	—0.7	±0	+1.8	+0.2
Serum from mice with SI ascites tumor with serum from mice with SI solid tumor	—3.9	+0.6	+0.9	—0.4	—2.7

* Figures in parentheses represent standard deviation of the mean.

** Corrected to level of serum protein.

TABLE IV

COMPARISON OF AMOUNT OF CARBOHYDRATE IN SI ASCITES TUMOR FLUID, SERUM FROM MICE BEARING SI ASCITES TUMORS AND SI SOLID TUMORS AND NORMAL MOUSE SERUM

a. Electrophoresis Data

Material analyzed	No. of samples	Mg per cc*					
		Total non-dialyzable carbohydrate	Albumin	α_1	α_2	β	γ
SI ascites tumor fluid	17	1.35 (0.20)	0.24 (0.06)	0.26 (0.07)	0.33 (0.07)	0.39 (0.13)	0.11 (0.43)
Ditto (corrected)**		1.57	0.28	0.30	0.39	0.46	0.13
Serum from mice with SI ascites tumor	7	1.32 (0.14)	0.23 (0.04)	0.19 (0.04)	0.40 (0.06)	0.38 (0.05)	0.11 (0.04)
Serum from mice with SI solid tumor	6	1.45 (0.26)	0.22 (0.03)	0.19 (0.08)	0.47 (0.07)	0.46 (0.13)	0.11 (0.03)
Normal serum	5	2.40 (0.21)	0.55 (0.05)	0.36 (0.13)	0.81 (0.15)	0.45 (0.03)	0.24 (0.07)

b. T Values

Fluids compared	T Value				
	Albumin	α_1	α_2	β	γ
SI ascites tumor fluid with normal serum	—10.8	—2.3	—8.3	—0.9	—5.0
Serum from mice with SI ascites tumor with normal serum	—8.6	—3.3	—6.6	—2.3	—3.9
Serum from mice with SI solid tumor with normal serum	—12.8	—2.7	—5.0	+0.3	—4.0
SI ascites tumor fluid with serum from mice with SI ascites tumor	+0.8	+3.2	—2.4	+0.2	+0.1
Serum from mice with SI ascites tumor with serum from mice with SI solid tumor	+0.5	—0.2	—1.9	—1.5	+0.2

* Figures in parentheses represent standard deviation of the mean.

** Corrected to level of serum protein concentration.

tion to filter paper. The loss during dialysis of carbohydrate is quite marked, about 50 per cent, in the case of both normal and tumor sera, as seen in Table V. The loss in dialyzable carbohydrate in the ascites fluid itself is significantly less, about 10 per cent. Electrophoretic and chemical analyses of the recovered dialysate showed that the dialyzable carbohydrates contributed to the orcinol value for total carbohydrate but did not stain on filter paper with the basic fuchsin Feulgen technique for protein-bound carbohydrate.

Discussion

Since the fluid of the ascites tumor is drawn from the peripheral circulation, deviations of the electrophoretic pattern of the ascites tumor fluid from that of serum of mice bearing the ascites tumor may be interpreted to be a result of tumor cell growth. Sarcoma I ascites tumor fluid was found to have a very distinctive electrophoretic pattern, showing five major components similar to those found in serum but quite different in proportions. The fluid contained more α_1-globulin protein and carbohydrate and more β-globulin carbohydrate than would be expected if it were composed entirely of serum, while α_2-globulin was decreased. A marked increase of α_1-globulin protein over that of serum has also been described by other workers in the ascites fluid from a patient with carcinoma (12).

It appears that beside adding protein and carbohydrate to their fluid medium, the ascites tumor cells may utilize substances from it for their growth. Sarcoma I ascites tumor fluid was found to contain only about 10 per cent dialyzable carbohydrate compared to 50 per cent in normal serum or serum from tumor-bearing mice. Our studies show also a lower total non-dialyzable or bound carbohydrate in both the ascites tumor fluid and serum from tumor-bearing mice than in normal serum, yet it is not proportionally lower in all the components. This is especially true in the case of the α_1- and β-globulin components of the ascites tumor fluid.

A possible explanation for these findings is that they represent a selective utilization of protein and carbohydrate for cell growth and a simultaneous addition of abnormal products as a result of cell metabolism. The work of various investigators supports this view. Klein and Klein (4) and Warburg and Hiepler (22) found that glucose concentration in the ascites tumor fluid decreased as the number of tumor

TABLE V

THE AMOUNT OF PROTEIN AND CARBOHYDRATE LOST DURING DIALYSIS OF SI ASCITES FLUID, SERUM OF MICE BEARING SI ASCITES TUMOR AND SI SOLID TUMOR AND NORMAL MOUSE SERUM

Material analyzed	Total Protein			Carbohydrate		
	Before dialysis	After dialysis	% dialyzed	Before dialysis	After dialysis	% dialyzed
SI ascites tumor fluid	49.58	44.34	10.57	1.36	1.06	22.0
	48.08	47.67	9.85	1.28	1.12	14.2
	50.90	50.00	1.79	1.72	1.63	5.2
	45.08	36.90	18.14	1.40	1.09	22.0
	44.16	43.45	1.83	1.22	1.13	5.7
			6.64			13.8
Serum from mice with SI ascites tumor	61.48	53.48	13.0	3.64	1.89	48.1
	62.18	53.78	13.5	3.68	1.38	62.5
			13.3			55.3
Serum from mice with SI solid tumor	78.86	75.96	3.68	4.52	1.32	70.8
	87.40	66.66	23.7	4.52	1.96	56.6
	71.40	65.70	7.98	3.20	1.54	51.8
			11.79			59.7
Serum from normal mice	65.14	62.40	4.21	3.35	1.73	48.4
	80.20	69.48	13.36	4.34	1.73	60.1
			8.78			54.3

cells increased. Henderson and Le Page (2) have reported that protein, per se, may be utilized by ascites tumor cells for growth. On the other hand, the presence of enzymes in the fluid, as discussed by Wu (25), may also account for carbohydrate utilization and protein addition.

The ascites tumor fluid would also be expected to reflect some of the serum changes found by many workers to accompany tumor growth (1, 19, 24), that is, a lowered albumin and increased α_2-globulin component. This raises the question as to whether the ascites tumor has the same effect on the serum components as does the solid form of the tumor. Our results show that in both Sarcoma I ascites tumor fluid and serum from mice bearing the ascites tumor, albumin is even lower than in mice bearing the solid form of Sarcoma I. They did not show, however, that α_2-globulin was increased over the normal as has been described by others using Tiselius electrophoresis (1, 19), but the β-globulin component was increased. This may be explained on the basis of a difference in mobilities of the proteins for the two techniques of electrophoresis. That different electrophoretic media result in changes in the relative mobilities of the protein components has been demonstrated (8).

The significance of these findings lies in the effect that these processes have on the tumor bearing host. Products of tumor cell growth liberated into the body may have toxic or otherwise adverse effects more harmful than the actual presence of the tumor itself. Their interference with the antibody mechanism may allow the growth of tumor cells to proceed in their characteristically uncontrolled fashion, or they may be responsible for the development of hypersensitivity associated with tumor growth (23). The present studies have approached these problems by examining ascites tumor fluid for evidences of tumor cell growth. Some have been found, and work is in progress to isolate and delegate biological properties to these substances.

Summary

To determine what effect the growth of Sarcoma I ascites tumor cells might have on the medium in which they grow, that is, the ascites tumor fluid, proteins and carbohydrates of the ascites tumor fluid were studied by chemical analyses and paper electrophoresis. The results indicate that both protein and bound carbohydrates are

added to the fluid as a result of tumor cell growth, while free carbohydrate is utilized by the tumor cells for metabolism. The electrophoretic pattern of the ascites tumor fluid was different from that which would be expected if it were made up entirely of serum, showing a larger amount of protein in the α_1-component and more carbohydrate bound to the α_1- and β-proteins than is found in serum from the ascites tumor-bearing mice. Dialyzable carbohydrate was only about 10 per cent of the total in the ascites fluid, compared to 50 per cent in normal serum or serum from animals bearing Sarcoma I in both the ascites and solid forms.

The ascites tumor fluid reflected some of the characteristic serum changes brought about by the growth of Sarcoma I, that is a lower amount of albumin and larger amount of β-globulin than is found in normal mouse serum. Serum from mice bearing the ascites tumor had even less albumin than did that from mice bearing the solid tumor, but this appeared to be the only difference in the effect of the growth of the two forms of the tumors on the serum proteins.

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STUDY OF PROTEIN AND ENZYME IN THE BRAIN OF DEVELOPING CHICK

R. H. Lee, P. U. Angeletti, F. G. Caramia

Zoology Department, Washington University, St. Louis, Missouri

In recent years electrophoresis has been widely used to fractionate and analyze the soluble proteins extracted from brain tissue; attempts have been made to correlate the changes observed in electrophoretic patterns and modifications in the physiological state of the cerebral tissue. (Lowenthal, Karcher Van Sande, 1959.) This type of work has been extended by using chromatographic techniques in order to provide greater resolving power and, more importantly, to allow the convenient isolation of many individual components for further characterization, particularly enzymes. This may allow the detection of more subtle differences among various patterns in the nature of their enzymes and other components than has been possible previously. The observations presented here on the chick brain are concerned with the changes in the soluble proteins fractionated by column chromatography on a cellulose ion exchanger which occur during development. In addition, a characteristic pattern of changes in the distribution of brain acid phosphatase will be shown.

Material and Methods

Embryos were removed from fertile eggs which had been incubated for 8 to 21 days at 37° C. and staged according to the Hamburger and Hamilton (1951) stage series from which equivalent age was calculated. Embryonic, newly hatched and adult chicken brains were removed and dissected clear from the membranes. All other structures such as optic cups were removed completely. The tissues were homogenized in Tris-phosphate buffer 0.005 *M* pH. 8.00 with a Vir-tis Homogenizer, and then centrifuged at 15,000 rpm. for 30 minutes in a refrigerated International centrifuge. The clear supernatant was dialyzed overnight against two changes of buffer and stored frozen at —20° C. until used for chromatography.

Column chromatography on DEAE cellulose was carried out as previously described (Moore and Lee, 1960; Angeletti, Moore and

Suntzeff, 1960); a 2% suspension of purified DEAE cellulose in Tris-phosphate buffer 0.005 *M* pH. 8.00 was packed in a 16 × 9 cm. column at 10 pounds pressure and washed by gravity for several hours in a cold room at 4° C. 10 ml. of extract, representing 40 to 60 mg. of protein, was applied to the column and allowed to soak in by gravity. After the initial washing with the starting buffer, a parabolic gradient salt elution was started to a final concentration of 1 *M* NaCl in 0.05 *M* phosphate buffer pH. 6.5. Fractions of 3 to 4 ml. were collected. Proteins were assayed by the Lowry method (1951). Optical density at 280 and 260 mμ was also measured in each fraction. Acid-phosphatase activity was measured by the Bessey-Lowry method (1946), using p. nitrophenylphosphate as substrate. Lactic-dehydrogenase (LDH), Isocitric-dehydrogenase (ICDH), and 6-phosphogluconic dehydrogenase (6-PGDH) were assayed by the conventional spectrophotometric techniques as reported in detail elsewhere. (Moore and Lee, 1960.)

Results

Fig. 1 shows typical chromatograms of soluble protein from brains of 10-day, 20-day and adult chicks respectively on DEAE cellulose.

It can be seen that a certain amount of protein did not bind to the column and was eluted by the starting buffer; this protein, having a probable 0 or negative charge under these conditions represented about 15-20 percent of the total proteins in all of the preparations. The remaining proteins bound to the column were then eluted by the gradient salt and fractionated into a number of peaks. The nucleic acids, evidenced by 260 mμ of absorbing material, were localized in a large peak at about 0.5 *M* Cl^- concentration. As shown in Fig. 1 the number of the protein peaks and their relative heights are somewhat different in the chromatographic pattern of the brain taken at different stages. When the protein eluted after the gradient salt began are considered, it can be seen that in the pattern of 10-day chick brain the amount of the most slowly and of the fast moving components relative to the intermediate components is higher than in the patterns obtained from older stages. As the brain matures, there is a decrease of the proteins loosely bound to the column and immediately eluted with the beginning of the gradient salt while the amount of proteins as well as the number of the separated peaks in

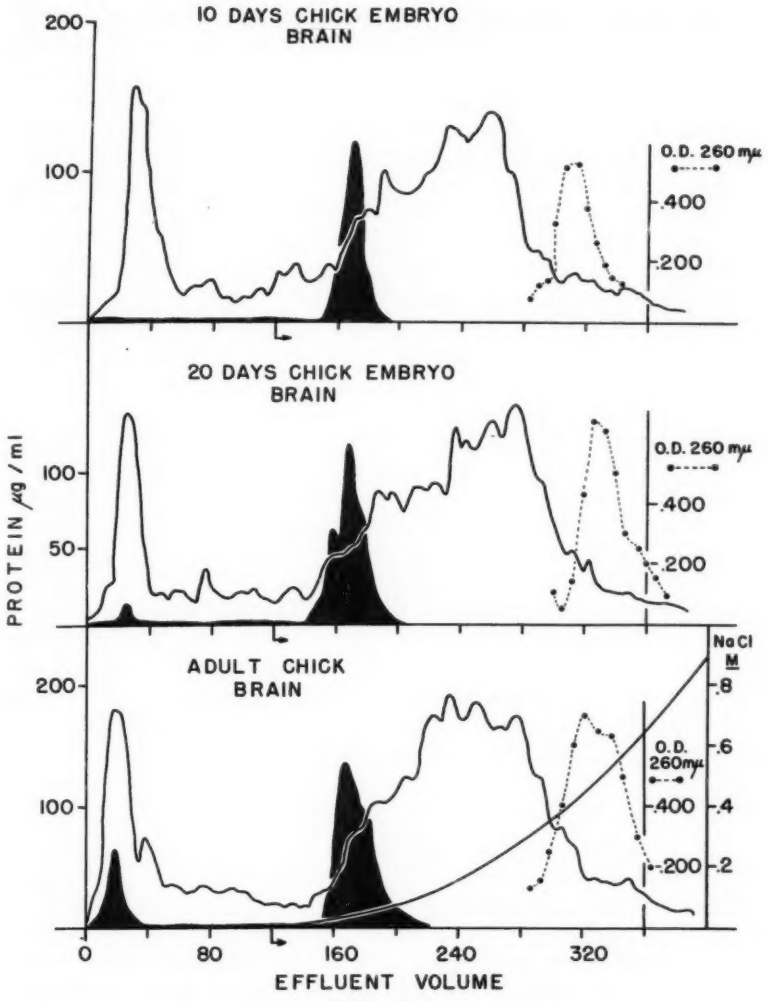

FIGURE 1

Chromatograms on DEAE cellulose of soluble proteins from chick brain at different developmental stages. Solid line: Protein; broken line: Optical density at 260 mμ; in black: Acid-phosphatase activity.

the intermediate zone of the chromatograms consistently increases. Of the enzymes tested, 6-PGDH and ICDH did not show significant changes in their pattern of distribution at the different developmental stages considered. In all chromatograms, 6-PGDH activity was consistently localized in one sharp peak at about 0.06 *M* Cl^- concentration; LDH and ICDH were separated in two zones, one associated with the first protein peak and another soon after the beginning of the gradient salt; for both enzymes, however, most of the activity (85 to 95%) was associated with the first peak. The acid-phosphatase activity on the other hand showed significant changes in its pattern of distribution during development of the brain. In the chromatograms of 10-day chick brain, the activity of this enzyme was localized in only one peak in a zone between 0.02 and 0.08 *M* Cl^-. In newly hatched embryos, the activity was still mainly localized in the same chromatographic region but traces of activity were also detectable in the first protein peak (Fig. 1). Finally, in the adult chick brain, the acid-phosphatase activity was consistently separated into two forms, one before and the other soon after the gradient salt elution. The first peak of the enzyme activity which appears in 20-day embryos accounts for about 20-30 percent of the total recovered activity in the adult. In experiments of rechromatography under the same conditions each single form of phosphatase activity was localized in the identical position in which it appeared during the first chromatographic fractionation (Fig. 2). Further characterization of these two forms of acid-phosphatase showed that they have different pH. optima (Fig. 3), different sensitivity to fluoride and tartrate inhibition (Fig. 4), and, finally, different Michelis constants (Fig. 5).

Discussion

The synthesis of new specific proteins is one of the main features of cell growth and differentation. The study of enzymes in development should, therefore, represent, as Brachet points out (Brachet, 1960) a useful approach toward a better understanding of the chemical modifications which underlie any structural and functional change in the tissues. The results presented above show that upon fractionation of brain soluble proteins by column chromatography, it is possible to reveal some important changes which take place within the tissues in different developmental states. There is evidence that the protein

pattern changes as the tissue matures, as indicated by the different chromatographic profiles; whether or not these changes are due to the appearance or disappearance of some particular components or simply to changes in their relative amount during development is a matter of further investigation. Experiments in progress (Moore, B. W., un-

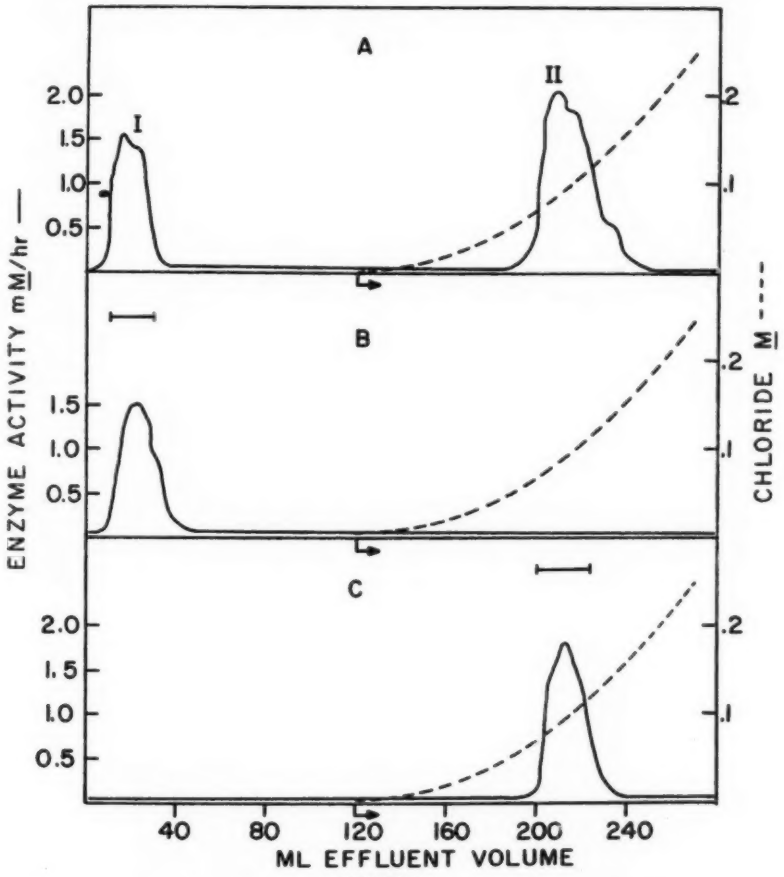

FIGURE 2

Rechromatography of each peak of acid-phosphatase from adult chick brain on DEAE cellulose. Solid line: Enzyme activity in micromoles/ml./hr.

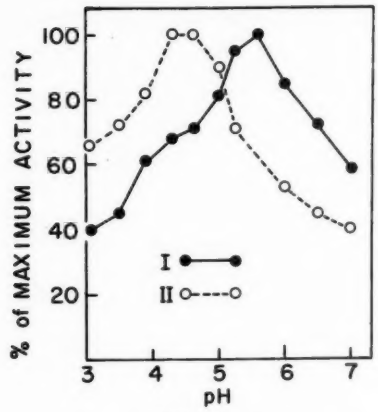

FIGURE 3

The effect of pH. upon the activity of acid-phosphatase separated in adult chick brain.

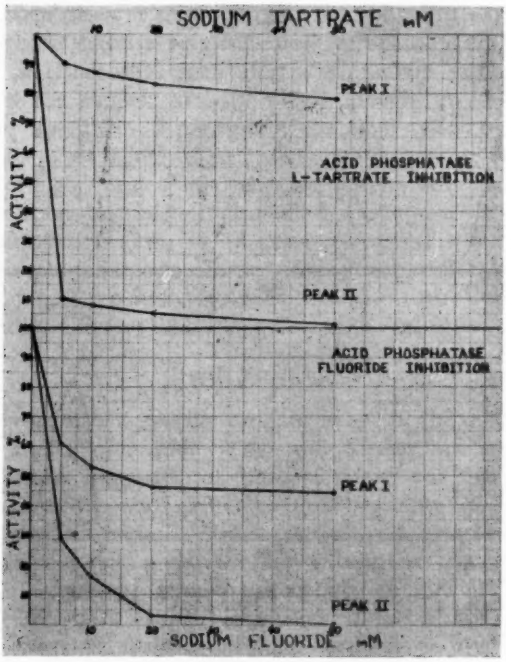

FIGURE 4

Inhibition of the two forms of acid-phosphatase in adult chick brain by fluoride and L-tartrate.

published) indicate that further resolution and characterization of brain soluble proteins can be achieved by applying starch gel electrophoresis to each single peak collected after column chromatography. Among the enzyme activities investigated, acid-phosphatase showed characteristic changes during development. This enzyme was, in fact,

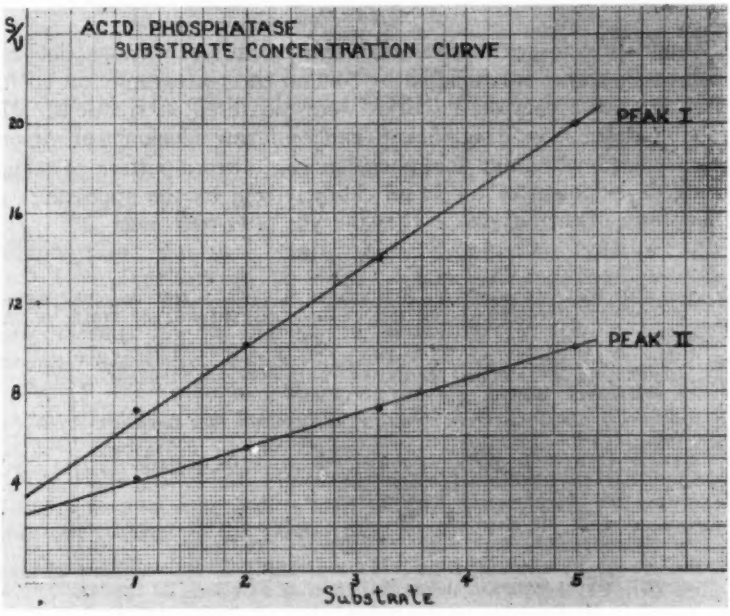

FIGURE 5

The effect of substrate concentration upon velocity. P-nitrophenylphosphate as substrate.

separated into two distinct forms in adult chick brain extracts, whereas only one peak of activity was found in brain extracts before hatching. The existence of multiple forms of an enzyme within the same tissue has been established by a number of investigations. (Markert and Moeller, 1959, Moore and Lee, 1960). There is also evidence that the pattern of distribution of multiple form enzymes may change during the tissue's growth and differentiation (Markert and Hunter, 1959), Flexner and co-workers, 1960). In our investigation, the

ontogenesis of brain acid-phosphatase in the chick appeared to be characterized by the appearance of a new form of the enzyme soon after hatching. The two forms of acid-phosphatase present in adult pattern differ from one another in their chromatographic as well as in some of their kinetic properties.

Summary

Soluble proteins extracted from chick brain at various developmental stages were fractionated by column chromatography on DEAE cellulose. Some enzyme activities were measured in fractions. The protein pattern showed significant changes during development. Among the enzymes investigated acid-phosphatase showed a pattern of change during development of the brain characterized by the appearance of a new form of the enzyme in the embryos after hatching.

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AN ONCOSTATIC FACTOR PRESENT IN THE BARK OF HIPPOPHAE RHAMNOIDES*

BORIS SOKOLOFF, M.D., KOJI FUNAOKA, PH.D.,
MICHIAKI FUJISAWA, M.D., CLARENCE C. SAELHOF, M.D.,
EIICHI TANIGUCHI, PH.D., LUIS BIRD, B.S.
AND CHARLES MILLER, B.S.

A. P. Cooke Memorial Cancer Laboratory, Florida Southern College, Lakeland, Florida

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In 1957, Pukhalskaya *et al.* (3) of the Institute of Experimental Pathology and Cancer Therapy, Moscow, reported isolation of an agent from the bark of an Asiatic plant, *Hippophae rhamnoides*, which inhibited Ehrlich mouse carcinoma. Pukhalskaya (4) furthermore described that an alcoholic extract of the bark, after elimination of tannic acid, gave LD_{50} 750 mg/kg for rats, intraperitoneal injection. A total dose of 400 mg/kg of the factor inhibited up to 80-90% of Guerin carcinoma, sarcoma 45 and sarcoma M-1, but no complete resorption of the tumors was observed. The oncostatic activity of the factor, called *hippophainum* was much less pronounced in regard to mouse hepatoma C_3HA, Ehrlich carcinoma, Passy melanoma and Walker rat carcinoma. Petrova *et al.* (1) by further chemical purification of hippophainum were able to increase its biological activity up to LD_{50} 250 mg/kg. Pukhalskaya *et al.* (5) identified the active factor as 5-hydroxytryptamine. They obtained it in the form of a hydrochloride. The daily doses, 10 mg/kg wt/mouse and 5 mg/kg wt/rat were found of low toxicity. Larger doses of hippophainum caused renal injury, affecting, however little, if at all, the blood forming system. The total dose, 60 mg/kg wt/mouse, given in 12 days, inhibited Ehrlich carcinoma by 42%. The same dose inhibited Guerin carcinoma by 67%. No inhibiting effect was observed on rat lymphosarcoma. No complete inhibition or resorption of any tested tumor was observed. Pukhalskaya (6) in her review of the pharmacology of

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hippophainum, which was identified by her as a hydrochloride serotonin, stressed its role in anaphylactic shock and as a vasoconstrictor. Petrova and Menshikov (*2*) described in detail the method of producing 5-hydroxytryptamine from the bark of *Hippophae rhamnoides*. They established that the dry bark contained 0.3-0.4% of this factor.

Material and Technique

The bark of *Hippophae rhamnoides* was obtained from the Institute of Experimental Pathology and Cancer Therapy, Academy of Medical Sciences, Moscow, U.S.S.R.** The hydrochloride 5-hydroxytryptamine was produced from the bark following the exact procedure described by Petrova and Menshikov (*2*). In brief, this procedure consisted in extracting the bark with 0.5% HCl, filtering, bringing the pH of the supernate down to 5. The material was passed through a column filled with cationic absorbant previously saturated with NaOH, and washed thereafter with distilled water and 5.0% HCl. Bicarbonate of soda was added to the eluate (pH 4.0) and dried in vacuum, in nitrogen atmosphere. The dry material so obtained was extracted with absolute ethanol several times and fractionated chromatographically (butyl alcohol-acetic acid-water system). The amorphous mass of hydrochlorides was dissolved in a small amount of absolute alcohol, acidified slightly (pH 6.2) with HCl and kept in a refrigerator at a temperature of 0° C. The hydrochloride 5-hydroxytryptamine was precipitated in the form of large crystals with a melting point of 167° C.

Mouse sarcoma 180, Ehrlich mouse carcinoma, and Murphy-Sturm rat lymphosarcoma, both solid and ascites tumors, were treated with various doses of hydrochloride 5-HT, injected intraperitoneally, for seven consecutive days. Although the Russian investigators used in their screening a five day tumor transplant, in our investigation, a one day tumor transplant served as a rule for the standard testing. After the treatment, the tumors were removed and their weights recorded. The original inoculum of ascites cells was about one million cells, and the number of cells was checked on the third, sixth and ninth day. The therapy was started six hours after the ascites cell inoculum.

** We wish to thank Dr. Eugenie Pukhalskaya of the Institute for the kind supply of the bark.

EXPERIMENTAL

Toxicity of hydrochloride 5-HT. 60 mice were divided into three groups. Group A, served as control. Group B received the total dose 60 mg/kg wt/mouse in seven days, and Group C was given 100 mg/kg wt/mouse in the same time period. Table I summarizes the results of this testing. While the mice of Group B lost weight only slightly as compared with the control, a total dose of 100 mg/kg wt/mouse was quite toxic to the animals, with a mortality rate of 25 %. Minute hemorrhages were observed in the lungs and kidneys of the mice receiving such an amount of hydrochloride 5-HT. When a total dose of 60 mg/kg wt of the drug was given to rats, they showed toxic side effects such as weight loss and sluggishness. Total dosage of 40 mg/kg wt/rat was non-toxic.

TABLE 1

THE CYTOTOXICITY OF HYDROCHLORIDE 5-HYDROXYTRYPTAMINE

Group A.—Control, 20 mice.

Group B.—Total dose, 5-HT, 60 mg/kg wt/mouse, given in 7 days, 20 mice.

Group C.—Total dose, 5-HT, 100 mg/kg wt/mouse, given in 7 days, 20 mice.

Groups	Number of mice	Original aver. wt. gm.	Aver. wt. after eight days gm.	Mortality rate %
Group A. Control	20	21.2	24.1	0
Group B. Total dose 60 mg/kg wt	20	20.2	22.1	0
Group C. Total dose 100 mg/kg wt	20	21.6	19.6	25

Antitumor activity of 5-HT. The total dose 60 mg/kg wt/mouse of hydrochloride 5-HT, administered in seven days, intraperitoneally, inhibited sarcoma 180, solid tumor, by about 60 %, and Ehrlich carcinoma by 35 %, as the figures of Tables II and III indicate. A larger total dose of the drug slightly augmented its oncostatic activity. Thus the total dose, 100 mg/kg wt/mouse, inhibited sarcoma 180, solid tumor, by 76 %, and Ehrlich carcinoma by 41 %, with some toxic side effects in evidence. The most striking fact was, however,

that a very small dose of 5-HT, such as 6.0 mg/kg wt/mouse, apparently enhanced the tumor growth. The average increase in tumor weight, both for sarcoma 180 and Ehrlich carcinoma was 9-10 %. As small as this increase was, it was recorded in all animals receiving such a total dose of 5-HT. Tables II and III illustrate these findings.

TABLE II

EFFECT OF HYDROCHLORIDE 5-HYDROXYTRYPTAMINE ON MOUSE SARCOMA 180 HARD TUMOR

Group A.—Control, no treatment, 20 mice.

Group B.—Total dose, 5-HT, 60 mg/kg wt/mouse, in seven days, 20 mice.

Group C.—Total dose, 5-HT, 100 mg/kg wt/mouse, in seven days, 20 mice.

Group D.—Total dose, 5-HT, 6 mg/kg wt/mouse, in seven days, 20 mice.

Groups	Number of mice	Aver. tumor wt gm after 8 days	Mortality rate %
Group A. Control	20	1.10	0
Group B. Total dose 60 mg/kg wt	20	0.45	0
Group C. Total dose 100 mg/kg wt	20	0.28	25
Group D. Total dose 6 mg/kg wt	20	1.19	0

The drug had very little inhibiting influence on Murphy-Sturm lymphosarcoma. The total dose, 40 mg/kg wt/rat given in seven days, inhibited the tumor by 17 %. The average treated tumor weight was 2.2 gm, as against 2.574 gm for the control. A larger total dose of 5-HT, 60 mg/kg wt/rat, produced no significant inhibitory influence: the average treated tumor weight was 2.0 gm, as compared with 2.38 gm of control. Much attention was given to administration of small doses of 5-HT. The series of tests with the total dose of 5.0 and 3.0 mg/kg wt/rat showed a certain enhancing effect of the drug on tumor growth. Thus with the total dose of 5.0 mg, administered in seven days, there was an increase in tumor weight of the treated group by about 8 % against the control group. For the treated group,

average 2.08 gm; and 1.925 gm for control. Each group consisted of 15 rats.

The same total dose, as used by us on solid mouse tumors, was applied to sarcoma 180 and Ehrlich carcinoma ascites tumors. The number of ascites cells and the survival time were recorded. When

TABLE III

EFFECT OF HYDROCHLORIDE 5-HYDROXYTRYPTAMINE ON EHRLICH MOUSE CARCINOMA HARD TUMOR

Group A.—Control, no treatment, 20 mice.

Group B.—Total dose, 5-HT, 60 mg/kg wt/mouse, in seven days, 20 mice.

Group C.—Total dose, 5-HT, 100 mg/kg wt/mouse, in seven days, 20 mice.

Group D.—Total dose, 5-HT, 6 mg/kg wt/mouse, in seven days, 19 mice.

Groups	Number of mice	Aver. tumor wt gm after 8 days	Mortality rate %
Group A. Control	20	1.01	0
Group B. Total Dose 60 mg/kg wt	20	0.66	0
Group C. Total Dose 100 mg/kg wt	20	0.59	20
Group D. Total Dose 6.0 mg/kg wt	19	1.12	0

the total dose, 60 mg/kg wt/mouse was administered to mice with sarcoma 180 ascites, the animals lived somewhat longer and the number of ascites cells, after completion of the seven day therapy, was about 25 % smaller than in the control group. Similar figures were obtained with Erhlich ascites carcinoma: 22 % of inhibition. With a larger total dose, 100 mg/kg wt/mouse, the inhibition was more pronounced: 33 % inhibition for sarcoma 180 ascites and 28 % for Ehrlich carcinoma ascites. The small total dose 6.0 mg/kg wt/mouse of 5-HT, somewhat stimulated the ascites cells multiplication, both of sarcoma 180 and Ehrlich carcinoma. The number of ascites tumor cells was increased by about 10 %. Tables IV and V present the results of testing.

TABLE IV

EFFECT OF HYDROCHLORIDE 5-HYDROXYTRYPTAMINE ON SARCOMA 180 ASCITES TUMOR

Group A.—Control, 20 mice.

Group B.—Total dose, 5-HT, 60 mg/kg wt/ mouse, in seven days, 20 mice.

Group C.—Total dose, 5-HT, 100 mg/kg wt/mouse, in seven days, 20 mice.

Group D.—Total dose, 5-HT, 6 mg/kg wt/mouse, in seven days, 20 mice.

Groups	Number of mice	No. of ascites cells on 9th day, aver. millions	Survival aver. time days
Group A. Control	20	834	11.2
Group B. Total dose 60 mg/kg wt	20	625.5	13.3
Group C. Total dose 100 mg/kg wt	20	562	12.5
Group D. Total dose 6 mg/kg wt	20	922	11.5

TABLE V

EFFECT OF HYDROCHLORIDE 5-HYDROXYTRYPTAMINE ON EHRLICH MOUSE CARCINOMA ASCITES TUMOR

Group A.—Control, no treatment, 35 mice.

Group B.—Total dose, 5-HT, 60 mg/kg wt/mouse, in seven days, 20 mice.

Group C.—Total dose, 5-HT, 100 mg/kg wt/mouse, in seven days, 20 mice.

Group D.—Total dose, 5-HT, 6.0 mg/kg wt/mouse, in seven days, 20 mice.

Groups	Number of mice	No. of ascites cells on 9th day, aver. millions	Survival aver. time days
Group A. Control	35	644	13
Group B. Total dose 60 mg/kg wt	20	502.8	16
Group C. Total dose 100 mg/kg wt	20	429	14
Group D. Total dose 6.0 mg/kg wt	20	712	12

No significant inhibiting influence of 5-HT was observed on Murphy-Sturm ascites lymphosarcoma, either with the total dose 40 mg/kg wt/rat or 60 mg/kg wt/rat. The survival time was almost the same as in the control: treated average, 13.5 days; control average, 12.5 days. The administration of a small total dose, 5 mg/kg wt/rat, slightly enhanced multiplication of lymphosarcoma ascites cells. The average number of ascites cells for ten treated rats was, after nine days, 851 million as compared with the control, 733 million cells.

The results of our screening tests on hydrochloride 5-hydroxytryptamine indicate that the drug, at certain concentrations, exerts an oncostatic influence upon sarcoma 180 and Ehrlich mouse carcinoma, in both solid and ascites forms. Thus our observations confirm those of Pukhalskaya *et al.* However, the drug had a much lesser effect on Murphy-Sturm rat lymphosarcoma, both solid and ascites tumors, than on mouse tumors. On the other hand, very small total doses of 5-HT, such as 5.0 or 6.0 mg/kg wt/mouse or rat seemed to have a stimulating effect on solid tumor growth and on ascites tumor cell multiplication.

Discussion

Scheline and Scott (8), Scott *et al.* (9), and Scott and Stone (10) reported that 5-hydroxytryptamine, used by them as the creatinine sulfate complex, enhanced moderately the growth of a rat sarcoma. The dose administered by them was about 9 mg/kg wt/rat. A study of iodide trapping by means of I^{131}, led these investigators to the conclusion that the iodide trapping syndrome appears to be mediated through a pathway involving 5-HT. "A parallelism exists," they assert "between iodide trapping and tumor growth. Increased tumor growth has been observed following the administration of 5-HT and histamine, as well as when these two compounds are released by destruction of mast cells with distilled water." The intrinsic mechanism of iodide trapping was not explained by them, but they expressed the opinion that since 5-HT release in tissue results in increased capillary permeability (according to Rowley and Benditt (7)), this may account for the loss of iodide and other substances and subsequently for the supply of nutrients to tumor cells. The whole problem of iodide trapping in its relationship to tumor growth, seems to be far from satisfactorily proved. There are some substances which cause iodide trapping but have definitely no enhancing influence on

tumor growth injected either in small or large doses to tumor-bearing animals (Scott and Strei (11)). Nevertheless, as far as 5-HT is concerned, our observations seem to confirm those of Scott *et al.* that small doses of this drug moderately enhanced tumor growth, while larger doses have a definite oncostatic influence, as reported by the Russian investigators.

Summary

A factor was isolated from the bark of *Hippophae rhamnoides* which was identified as hydrochloride 5-hydroxytryptamine.

A total dose of 60 mg/kg wt/mouse, 5-HT, administered over seven days, inhibited sarcoma 180 by 60 % and Ehrlich carcinoma by 35 %.

Total dosage of 100 mg/kg wt/mouse, 5-HT, inhibited sarcoma 180 by 76 % and Ehrlich carcinoma by 41 %, but induced toxic side effects.

Total dosage of 6.0 mg/kg wt/mouse, 5-HT, enhanced slightly the growth of sarcoma 180 and Ehrlich carcinoma, both solid and ascites cell tumors.

The factor had no significant inhibiting effect on Murphy-Sturm rat lymphosarcoma.

The possible role of 5-HT in iodide trapping is discussed.

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BOOK REVIEW

Nomina Anatomica. 2nd ed. Excerpta Medica Foundation, Amsterdam, 1961. 99pp.

This is a standard list of anatomical names, compiled and authorized by the International Anatomical Nomenclature Committee, with amendments and corrections approved by the Seventh International Congress of Anatomists, New York, 1960. According to the introduction, 104 terms have been changed, the positions of 58 rearranged, and 119 new terms have been added. Inconsistencies, grammatical errors, and incorrect spellings appearing in the first edition have been corrected.—

Ed.

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ERRATUM

Addition and correction to REYMENT, R. A. "A Note on Geographical Variation in European *Rana*," GROWTH, September, 1961, pp. 220-221.

Page 220. Vector $\bar{x}_1$ is that for females ($N_1 = 35$) and vector $\bar{x}_2$ is that for males ($N_2 = 14$).

Page 221. The matrix U should read

$$\begin{bmatrix} 16861.24 & 8862.01 & 835.19 \\ 8862.01 & 5094.31 & 461.98 \\ 835.19 & 461.98 & 69.82 \end{bmatrix}.$$

I am grateful to Pierre Jolicoeur, Montreal, for drawing my attention to these points.

R. A. Reyment